A Methodological Investigation of Production, Purification and Assay of Monoclonal and Polyclonal Antibodies and their Utilisation for Quantitative, Comparative and Functional Analysis of Components of the Eukaryotic Nucleus

DISSERTATION

zur Erlangung des Grades eines Doktors
der Naturwissenschaften
der Fakultät für Biologie
der Eberhard-Karls-Universität Tübingen

vorgelegt
von

PETER SYMMONS
aus London, Vereinigtes Königreich
von Großbritannien und Nord Irland

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Tag der mündlichen Prüfung	23. Februar 1984
Dekan	Prof. Dr. E. Kulzer
1. Berichterstatter	Prof. Dr. P. Hausen
2. Berichterstatter	Prof. Dr. V. Hemleben

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INTRODUCTION

Within the nucleus of the eukaryotic cell reside the molecular systems responsible for DNA replication, for the selective transcription of RNA from a DNA template and for the selective transport of transcribed sequences to the cytoplasm. These systems are complex: two dimensional gel electrophoretic analysis of the protein composition of the nucleus reveals that it is made up of at least 1000 polypeptides varying widely in abundance (PETERS and COMMINGS 1980, FELDHERR and OGBURN 1980). Many of these polypeptides are confined to the nucleus by (an) as yet unknown mechanism(s) (GURDON 1970, De ROBERTIS et al. 1978, FELDHERR & OGBURN 1980, DREYER et al. 1982, ZELLER et al. 1983, DEQUIN et al., submitted). A set of small RNA molecules of uncertain function is similarly confined to the nucleus (BUSCH et al. 1982, ZELLER et al. 1983).

Given this level of complexity, it would not be feasible to unravel the function of any but a very small number of these polypeptides using the classical biochemical techniques of fractionation, purification and reconstitution. A particular problem would be the vast amount of material required for characterising the rarer components. To take Drosophila melanogaster as an example: if only 10 molecules of a particular polypeptide of molecular weight 20,000 daltons were to be present per nucleus, then the purification of 10 µg of this component would require the production of 10,000 litres of cultured cells or about 250 kg of intact adult organisms (assuming a recovery of 40% and a generous estimate of the equivalent of 10^6 diploid nuclei per intact animal).

An alternative approach to the molecular analysis of the nucleus, which avoids this problem, is to use the immune system of small mammals to provide the necessary tools. The immune system of these animals is capable of producing more

than 10^7 different antibody molecules each with different ligand-binding properties. Clones of the cells known as B lymphocytes each produce a single kind of antibody. The size and number of B-cell clones is subject to a complex reticulate system of controls (JERNE 1976), but what is relevant here is that it may be reset by introducing as few as 10^{11} foreign molecules (approx. 1 ng of a 20,000 MW polypeptide) into the system (LUBEN et al. 1982).

Hybridoma technology (KÖHLER & MILSTEIN 1975) now allows a single clone producing a single antibody - a monoclonal antibody - to be propagated indefinitely outside its original progenitor. Provided that clones producing antibodies against nuclear components can be induced with sufficient frequency, it is now possible to produce monoclonal antibodies as markers for almost all nuclear components (SAUMWEBER et al. 1980, RISAU et al. 1981, TURNER 1981, DREYER et al. 1983, MÖLLER et al. 1982). The nuclear location and/or functional association of nuclear components can now be examined by following them with their specific marker antibodies. The large scale identification of which molecular component is involved in which nuclear function becomes trivial. Attention can now be focussed on making analyses more precise, and in principle it should be possible to obtain information about the quantitative composition of the nucleus.

In practice, however, there are a number of problems, some technical, some theoretical which must be solved before a complete immunological dissection of the nucleus can be achieved. Their nature and some partial solutions are the subject of this dissertation.

Many of the problems arise because much immunological methodology was initially developed for use with polyclonal antibodies (i.e. mixtures of antibodies, usually directed against a collection of different ligands on a particular

macromolecule) rather than with monoclonals (a single antibody against a single ligand). In the polyclonal mixtures differences between individual antibodies (e.g. charge, idiosyncratic chemical or solubility properties) were averaged out, whereas between monoclonals the occurrence of these differences is unavoidable. Problems arising from differences between antibodies are further exaggerated by the use of the mouse as the source animal for monoclonals, since the mouse produces 4 different subclasses of IgG antibodies (non-allelic variants of the constant region of the antibody) with different biochemical characteristics which are of considerable practical importance. By contrast the rabbit, a traditional source of polyclonal antibodies, produces only one class of IgG molecule. Methods for quantitating antigen by counting the antibody molecules bound to them were more or less non-existent until monoclonal antibodies came into vogue. Immunological quantitation was until recently based almost exclusively upon competition for binding to solid-phase bound antibody between unknown amounts of an unlabelled antigen and a standard amount of labelled antigen (PARKER 1976).

The monoclonal antibodies whose behaviour I have investigated in this study are very powerful tools which will still be available for many years to come and can therefore be used as standards. I have devised methods for their mass production and have isolated some in highly purified form (chapters R.1 to R.4). Although the properties of only a few of the antibodies which were potentially available to me have been examined, the results which I have obtained do demonstrate that there are differences between them and that they must be used with care in order to avoid generating artefacts (chapters R.4 and R.7).

I have thoroughly investigated some of the properties of the solid phase assay routinely used by our research group (chapter R.7) and show that when **mixtures** of antigens are

adsorbed to a solid phase, under conditions where the antigen is in excess of the binding capacity of the solid phase, the outcome of the competition between antigens is unpredictable. This prevents the method from being used to provide reliable quantitative estimates of antigen frequency. However, by making some plausible assumptions, and by making use of estimates of antigen abundance provided by RISAU (1983), I argue in chapter R.9 that, under favorable circumstances, the assay should be able to detect Drosophila melanogaster nuclear antigens with a frequency of as little as 10^3 copies/diploid nucleus. I discuss improvements to this convenient and tested assay which should allow even the hypothetical 10 copy/nucleus polypeptide used in the example at the beginning of this introduction to be detected.

In addition to demonstrating and solving some of the problems associated with the production, purification and assay of antibodies (part I of the thesis), I also present results of two novel applications of immunological techniques for characterising nuclear antigens.

The first is a quantitative histological procedure, using immunofluoresence, which allows the spatial and quantitative distribution of two antigens in the nucleus to be examined simultaneously (chapter R.10). The correlation (or lack thereof) between these two parameters allows inferences to be made about whether pairs of antigens are likely to be involved in the same or different nuclear functions.

The second is the serendipitous discovery that the immunological reactivity of the nuclear protein histone H1 changes on binding to supercoiled DNA (chapter R.11). This finding indicates that it might be possible to use immunological methods (albeit indirectly) to investigate topological properties of nucleic acids.

Taken together, I hope that the results presented in this thesis will convince the reader of the versatility and power

of immunological methods for increasing our knowledge of the eukaryotic nucleus. At the same time I hope that he or she will also be made aware of the care which must be exercised to ensure such methods are reliable.

METHODS and MATERIALS

M.1 Analytical Methods

Most of the methods described in section M.1 are established and in general use in biochemical laboratories. However, many minor variants of these methods exist, some of these are to be found in the literature, but others are often simple adaptations for local conditions e.g. altering volumes so that a particular kind of tube can be used; furthermore, the various alterations and improvements are often used in combination. Rather than just compiling a list of references to document the precise form of the test I have used, I have described, in full, the actual procedure employed, so that a reader of this work can use it directly, as well as a source of reference. In addition, where it is relevant, the characteristics and limitations of particular methods will be described here rather than in the "Results" section.

M.1.1 Quantitative Protein Determination.

M.1.1.1 Bradford Method

This method is the "microprotein assay" originally described by BRADFORD (1976).

Reagent Solution

To make 1 l: to 100mg Coomassie Blue G250 was added a mixture of

50 ml 95% Ethanol
100 ml 85% Phosphoric Acid

with stirring. After dilution to 1 l with destilled water the reagent was stored in a light-protected bottle with a tight fitting stopper. Under these conditions and stored at room temperature the reagent gave identical results with standards for a period of 6 months.

Assay

To measure 1 - 20 µg protein in the concentration range 10 µg/ml - 200 µg/ml

To 100 µl of the solution to be assayed 1 ml of Reagent solution was added. The mixture was briefly vortexed and allowed to stand for 5 minutes at room temperature. The O.D. at 595 nm was measured in a Spectrophotometer against a blank containing reagent and buffer (Buffer blank). The difference in absorbtion between test and blank $\Delta O.D._{595}$ was calibrated using a dilution series of a solution of a purified protein whose concentration had been determined by direct spectrophotometric measurement (see M.1.1.2). For some determinations the assay was carried out in microtitre plates and was read automatically using a Dynatech MR580 microelisa reader measuring at 630 nm against 405 nm (RYLATT and PARISH 1982). For these determinations the above volumes were reduced by a factor 5.

M.1.1.1.1 Limitations of this method

The method estimates protein by making use of a shift in the absorbance spectrum of the dye upon complexing to proteins. The absorbance of the uncomplexed dye at 595 nm under the conditions used here is already high - approx 0.65 O.D.- so the sensitivity of the method is limited. Furthermore as the curves in Fig 1 show, for ΔO.D values exceeding 0.4 the dependence of the spectral shift upon the amount of protein deviates from linearity; measurements can of course be restricted to the linear range by using appropriate dilutions. However, this is not the major limitation of the method, but rather the fact that the dye gives different results with different proteins. This is shown in Fig.1 for

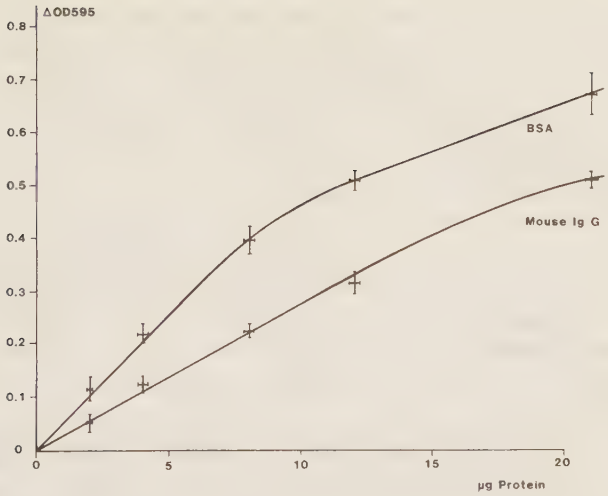

Fig. 1 **Calibration curves for the Bradford dye-binding protein assay using two different protein standards.**
The assay was performed as described in the text using stock solutions of each protein whose concentration had been determined spectrophotometrically. The error bars in the vertical direction show the measured Standard Error (S.E.) of 4 independent determinations and contains a contribution from the variation in the blank. The horizontal error bars indicate the errors which would occur with a pipetting error of ±0.5 µl below 10 µl and of ± 1.0 µl in the range 10-100 µl. Curves were fitted by inspection.

two proteins (Mouse IgG and BSA) between which, in the linear portion of the graph, there is a difference of almost a factor 2 in the extent of the absorbance change when identical amounts of protein are used. This is in agreement with a number of reports in the literature (ANON 1980, PIERCE and SUELTER 1977, VAN KLEY and HALE 1977, READ and NORTHCOTE 1981), in which a variation in the absorbance change between proteins as large as a factor of 5 has been observed. This is further documented in Table 1. This shows the results of an experiment in which known amounts of each of twenty purified proteins were tested using three different methods. Both the range and the Standard Error (S.E.) of the estimate of the amount of protein is greater by a factor of about 2 using the

Bradford method compared to the other two methods.

	METHOD OF ESTIMATION		
	Bradford	Lowry[1]	Biuret[2]
Mean amount of protein estimated (relative to IgG)	1.15	0.81	0.94
S.E.	0.5	0.25	0.18
Range	0.51-2.64	0.42-1.35	0.62-1.26
Range expressed as ratio of highest/lowest	5.2	3.2	2.0

[1] LOWRY et al. (1951) [2] see M.1.1.3

Table 1 **Variation between estimates of the amount of protein in samples containing identical amounts of different purified proteins using different colorimetric methods**
For each of the three tests the value obtained for IgG (source unspecified) has been set at 1.00: the values obtained with the other proteins are expressed relative to this. A set of parameters describing the data set for each of the methods has been calculated and is tabulated below. The data relates to 20 arbitrarily selected purified proteins and was obtained from ANON (1980).

It follows from these observations that the BRADFORD method is unsuitable for estimating the absolute amount of protein in unknown mixtures, unless some independent measure of the absolute amount of protein is available.

Variation between Bradford estimates appears to be caused by differences in the stoichiometry of dye binding to protein. The spectral shift in the absorbtion maximum of the dye on protein binding is identical for a number of proteins (PIERCE and SUELTER 1977, READ and NORTHCOTE 1981), but for equal weights of protein light absorbtion at this maximum varies.

Despite the major limitation of this method, it still useful for obtaining either rough estimates of the amount of protein in a sample, or for estimating the relative amounts of protein in series of samples containing either unknown amounts of a particular purified protein or protein mixtures of similar composition. A major advantage of this test is its

extreme simplicity, and its insensitivity to interference by non-proteinaceous substances (BRADFORD 1976), and this is the reason why it has been employed in most instances rather than more reliable, but much less sensitive, Biuret method (M.1.1.3), or direct spectrophotometric determinations.

READ and NORTHCOTE (1981) have published a thorough study of the Coomassie Blue G dye-binding assay, in which many of the comments I have made above are echoed. In addition, these authors claim to have found conditions for dye-binding under which the variation in dye-binding between different proteins is considerably reduced with respect to that found using the method described here. However, in my hands their procedures often led to the precipitation of protein and to erroneous readings as a result of light scattering from the precipitate. Therefore for most protein determinations I made using the BRADFORD method I continued to use the original procedure.

One further point should be made: given the variation which is observed with the form of this assay that I used it might be extremely misleading to express results obtained using it simply in terms of "µg protein". Therefore the convention is used of expressing results in terms of "µg equivalents of 'protein X'", where X is the name of the protein used to calibrate that particular assay. Conversion factors are listed in table 2 below, which tabulates the ratio between the slopes obtained for different proteins in the linear portion of their calibration curves.

		Protein 2		
		BSA	Mouse IgG	CT Histones
	BSA	1.00	1.86	1.27
Protein 1	Mouse IgG	0.54	1.00	0.68
	CT Histones	0.79	1.46	1.00

Table 2 **Conversion table for Bradford protein estimation using different standards.**
Factors to convert values expressed in terms of "µg equivalents of protein 1" to "µg equivalents of protein 2".

i.e µg equiv protein 1 x factor = µg equiv protein 2

- -

M.1.1.2 Determination of Protein Concentration by spectrophotometric determination of ultraviolet absorbance.

The extinction coefficients at two ultraviolet wavelengths for neutral aqueous solutions of the purified proteins used as standards in this work are tabulated in Table 3.

M.1.1.3 Biuret Method

This is an extensively modified version of the procedure originally described by GORNALL et al. (1949).

Reagent Solution

9.0 g K,Na Tartrate was dissolved in 400 ml distilled water. To this solution was added:

3.0 g $CuSO_4.5H_2O$
8.0 g NaOH
5.0 g KI

and the solution made up to 1l with distilled water. The solution was stored in screw top bottles and was stable for several months at room temperature.

Protein	wavelength (nm)	$\varepsilon^{1\%}_{1cm}$	mg/ml giving 1 O.D	Reference
BSA	280	6.6	1.51	1
	230	52	0.195	2
Histone 1	280	2.0	5	2
	230	20	0.5	3
Core Histones	280	4.25	2.35	2
	230	42.5	0.235	4
IgG (Mouse)	280	14.0	0.714	5
	230	72.6. (71.1-74.9)	0.137 (0.134-0.141)*	2
Lysozyme (hen egg)	280	26.4	0.38	6
	230	-	-	-

* This was the range using a panel of six purified monoclonal antibodies

Table 3 **Extinction Coefficients of Protein Standards**

References

1) TANFORD and ROBERTS (1952) 2) This work
3) RENZ and DAY (1976) 4) OHLENBUSCH et al. (1967)
5) There does not appear to be a published value for Mouse IgG (per se). This value is the one assumed by the commercial supplier MILES. The mean value for the $\varepsilon^{1\%}_{1cm}$ from 6 other mammalian species (data taken from FASMAN 1976a) is 13.64 (SE 0.53). The $O.D._{280}$ of monoclonal IgGs will in any case not be uniform because of the different amino acid compositions of the variable regions. However, since constant region amino acid composition of mouse IgG is known to be similar to that of other mammals, (HOOD et al., 1978 p.220; FASMAN 1976b p.403 ff.) it seems reasonable to think that a minimal amount of error will be introduced by making this assumption.
6) AUNE and TANFORD (1969)

Assay

To measure 0.5 - 4.0 mg protein in the concentration range 0.5 mg/ml - 5.0 mg/ml.

To an aliquot of protein solution containing approx. 1 mg protein 1/8 volume 3M Trichloracetic Acid was added. The flocculent mixture was stood on ice for 2 hours and then centrifuged in an Eppendorf Table Centrifuge (fixed angle, mean radius 4.75 cm) at 8000 rpm for 2 minutes. The supernatant solution was discarded and the pellet allowed to drain thoroughly. 1 ml of reagent solution was added and the sample vortexed until the pellet dissolved and then stood for at least 30 minutes at room temperature before the colour was read at 546 nm.

Some crude cell extracts gave rise to particularly compact pellets, which did not dissolve following a single vortexing. Vortexing repeatedly at 30 minute intervals over a period of approximately two hours solved this problem. Once the colour had developed it was stable for several days at room temperature. Concentrations of unknown proteins were determined by comparison with a calibration curve obtained from a standard whose concentration had been determined from its ultraviolet absorbtion (see M. 1.1.2).

M.1.2 Quantitative Determination of DNA

M.1.2.1 Diaminobenzoic Acid (DABA) Method

This method, originally descibed by KISSANE and ROBINS (1958), has been used to directly determine the amount of DNA in intact tissue and in crude cell extracts. This is possible because 3, 5 diaminobenzoic acid reacts with 2-deoxy sugars to

form highly fluorescent derivatives. Although the reaction is a general one for aldehydes in which the carbon atom adjacent to the aldehyde is unsubstituted, deoxyribose is the only cellular component present in any quantity with these properties, and in acid precipitable form deoxyribose is only to be found in DNA.

Because ribose is substituted at the 2 position, it produces negligible interference and DNA may therefore be assayed even in the presence of excess RNA: it has been shown that an equal mass of ribose gives a signal level less than 1% that of deoxyribose (KISSANE and ROBINS 1958, THOMAS and FARQUHAR 1978). Furthermore, proteins and lipid have also been shown to give rise to negligible interference (KISSANE and ROBINS 1958) as have a number of other non-DNA substances such as detergents (THOMAS and FARQUHAR 1978). The only frequently encountered substance which might seriously interfere with estimates of DNA is sucrose, which also gives rise to fluorescent derivatives, albeit with a yield approx. 1/200 that for deoxyribose (THOMAS and FARQUHAR 1978). In order to eliminate problems such as this, and also those arising from light scatter, which could be present if particulate cell fractions were assayed, or from a possible contribution of a free deoxyribonucleotide pool, a modification of the original method (ERWIN et al. 1981) was employed. In this variant DNA (and protein) is first acid precipitated, allowing the removal of almost all low molecular weight materials, and DNA is then quantitatively solubilised (presumably to a mixture of purines, deoxyribose, deoxyribose phosphate, pyrimidine nucleotide phosphate and diphosphate - [see BROWN and TODD 1955 p442ff]) by hydrolysis with hot (90^{o}C) TCA, and can thus be separated from cell protein, most of which remains precipitated.

Reagent solutions

Trichloracetic acid 5% w/v (0.31M)

DABA 200mg/ml in distilled water
(prepared immediately before use)

The DABA usually formed a light brown solution; such solutions gave high backgrounds. To reduce this, solutions were decolorised using active charcoal (Norite): for each 10 ml of solution 1 spatula tip of active charcoal (heated at 100 ^{o}C 1hr before use and used immediately) was added. The mixture was thoroughly shaken and allowed to stand for 5 minutes at room temperature. Active charcoal was removed by centrifugation (several centrifugations were often neccessary). The resulting solution was colourless.

Assay Procedure

To 200 µl of a suspension or a solution of DNA containing material (DNA content 0.5 - 5 µg) was added 1 ml 5% TCA, the mixture was whirlmixed and stood on ice for at least 4 hr (usually overnight). The resultant precipitate was centrifuged in an Eppendorf table centrifuge (fixed angle, mean radius 4.75cm, 8000 rpm) for 2 minutes to form a hard-packed pellet. This pellet is often translucent and therefore difficult to see. The supernatant was sucked off using a pasteur pipette attached to a vacuum pump. Care was taken to avoid mechanical disturbance of the pellet by the pipette. Because of the pellets' poor visibility this was done by orienting the assymmetric centrifuge tubes in the centrifuge such that the side on which the pellet formed could always be identified; the pasteur pipette then only

made contact with the tube wall on the side opposite to the pellet. Surface tension sufficed to allow complete draining of the pellet. If the DNA containing material also contained sucrose, then the pellet was washed with a further 1 ml 5% TCA by vortexing, allowed to stand for 1hr on ice and then handled as above. 500 µl 5% TCA was added and the tubes were heated at 90°C for 1 hour.

The tubes were allowed to cool, centrifuged briefly (to recombine the condensate on the lid of the tube with the rest of the reaction mixture) and 400 µl of supernatant was transferred to a fresh tube (to separate hydrolysed nucleotides from the pellet). 200 µl DABA reagent solution was added, the tubes were vortexed and then incubated at 60°C for 1hr. The reaction was ended by removal from heat and addition of 200 µl 5M HCl.

Fluoresence was measured using a 500 µl cuvette in a Perkin-Elmer model 3000 Spectrofluorimeter using excitation and emission slits at setting 10. Excitation was at 400nm and emission was measured at 500nm. For measurements using amounts of DNA less than 1 µg it was necessary to operate the instrument with nearly maximum amplification of the fluoresence signal. At these high amplification values, the signal was very sensitive to the position of the cuvette within the instrument. In order to obtain reliable results it was neccessary to make measurements using only one of the four available positions on the rotatable cuvette carriage, since it proved extremely difficult to align the carriage precisely after it had been rotated. Carefully lifting the cuvette in and out of the carriage did not affect readings, even when this procedure was repeated several times. The measured values are the arithmetic means of 100 individual 1 second readings. Excitation and emission spectra of the DNA-DABA complex were recorded and showed

that 400 and 500 nm were the peak wavelengths of excitation and emission respectively (results not shown) and use of this wavelength pair obviously maximised the fluorescence signal from the complex. Other authors (SETARO and MORLEY 1976) have used different wavelength pairs. No attempt was made to find out the reason for this discrepancy. It seems unlikely that the absolute calibration of the spectrofluorimeter can have been greatly in error since no such discrepancy was observed using ethidium Bromide. Earlier authors may have been limited in the wavelength pairs they were free to use.

M.1.2.1.1 Limitations of this method

The fluoresence is not completely stable, and in all samples, including the buffer control, appeared to increase at a constant absolute rate, about 3% of the initial value of the buffer blank per hour (?as a result of exposure to air?). Provided the signal to background ratio is large and the samples are measured quickly the problem can be neglected. If however signal levels are low, and large numbers of samples must be measured, it may be neccessary to monitor the time course of the increase accurately and to correct measurements appropriately.

More sensitivity has been obtained using other variations of the original method (SETARO and MORLEY 1976, THOMAS and FARQUHAR 1978), and it has been shown that increased sensitivity can be simply achieved by allowing the DABA deoxyribose reaction to take place under neutral rather than acid conditions (SETARO and MORLEY 1976). Nonetheless, even the insensitive variant described here is approximately 10x more sensitive than colorimetric reactions for DNA determination (GILES and MYERS 1965, SETARO and MORLEY 1977) which are still commonly used (cf e.g. LENGYEL et al 1975).

M.1.2.2 Fluorimetric Determination of DNA using Ethidium Bromide

This method makes use of alterations in the optical properties of ethidium bromide which occur when it forms complexes with nucleic acids. Whereas the free dye has an absorption maximum by 480nm in aqueous solution and is only slightly fluorescent, the nucleic acid complexed form has an absorption maximum at 530nm (WARING 1965) and the fluoresence is enhanced by a large factor (LePECQ and PAOLETTI 1967). It is the fluoresence properties of the complexed dye which allow it to be used for the estimation of submicrogram amounts of nucleic acids. However, whereas it is a trivial matter to use the dye's fluoresence to assay purified DNA, any attempt to assay DNA in crude cell extracts poses a number of problems. Firstly ethidium bromide can interact with subcellular organelles to give rise to a fluorescent signal (see e.g. LAYTON et al. 1973), secondly RNA will also contribute to the signal (see M.1.3.1), thirdly proteins binding to DNA limit its accessibility to the ethidium ion (PAOLETTI et al., 1977; KARSTEN and WOLLENBERGER 1972) and fourthly, suspended material could give rise to light scattering artefacts when fluoresence is measured.

KARSTEN and WOLLENBERGER (1972) showed that these problems could be overcome simply by making a sufficiently fine homogenate of cells and by digesting away RNA and Protein with RNase and protease respectively. Light scattering can be corrected by use of the appropriate blanks (KARSTEN and WOLLENBERGER 1977).

Reagents

		g/l	mM
DPBS	NaCl	8.0	137.0
	KCl	0.2	2.7
	KH_2PO_4	0.2	1.5
	$Na_2HPO_4.2H_2O$	1.15	6.5
	$MgSO_4.7H_2O$	0.1	0.41
	$CaCl_2.2H_2O$	0.1	0.68
	pH adjusted to 7.1		

RNase	200 µg/ml in DPBS
Proteinase K	200 µg/ml in DPBS
Ethidium Bromide	25 µg/ml ($6.34\ 10^{-5}$M)

The molarity of the ethidium bromide stock was checked by assuming a molar extinction coefficient at 480 nm of 5600 for a 1 cm pathlength cuvette (WARING 1965)

Sample Preparation

In order to obtain homogeneous samples cells and all crude cell fractions were sonicated extensively (6 x 10 seconds on ice with approx. 1 minute pauses between sonications) using a Branson sonifier with microtip - setting 5 - 7.

Assay Procedure

The following reaction mixture was made up:

DNA containing material	100 µl
Proteinase K	100 µl
DPBS	500 µl

mixed several times by inversion and incubated at 37°C in a water bath. After 30 min

RNase 100 µl

was added to each tube and the incubation continued for a further 30 min. The samples were then removed from the water bath and allowed to cool.

ethidium bromide 200 µl

was added to each and mixed by vortexing.

Identically treated tubes in which DPBS replaced ethidium bromide or sample served as light scattering and fluorescence blanks respectively. The final concentration of ethidium bromide was 5 µg/ml ($1.27.10^{-5}$M).

Fluoresence was measured using a 500 µl cuvette in a Perkin-Elmer model 3000 Spectrofluorimeter using excitation and emission slits at setting 10. Excitation was at 531nm and emission was measured at 593nm. For measurements using amounts of DNA less than 1 µg it was necessary to operate the instrument with nearly maximum amplification of the fluoresence signal and to take all the precautions described in Section M.1.2.1 to ensure that the light path through the cuvette did not change. The measured values are the arithmetic means of 100 individual 1 second readings. Excitation/emission wavelengths were determined empirically by recording spectra with pure DNA standards. The 530 peak corresponds to the known absorbtion maximum of the DNA-ethidium complex (WARING 1965); the emission peak is a very broad one varying less than 10% between 590 and 605nm (results not shown). Higher signal levels were attainable from pure DNA using excitation at 280nm, but using this wavelength with crude cell extracts gave rise to highly variable results, probably because of light scattering. No such

problems were encountered using 530 nm as the excitation wavelength. Fluoresence was calibrated by using identically treated samples containing known amounts of purified bacteriophage lambda DNA (Miles), from a stock solution whose concentration had been determined spectrophotometrically at 260nm assuming $\varepsilon^{1\%}_{1cm}=200$.

DNA fluoresence F_{DNA} was calculated as follows:

$$F_{DNA} = F_{sample} - (F_{sample\ blank} + F_{fluoresc.\ blank})$$

M.1.3 Fluorimetric determination of RNA using Ethidium bromide

The principle of this method is the same as that used for the determination of DNA: the enhancement of fluorescence of the ethidium ion upon interaction with nucleic acids. The extent of the enhancement is smaller for RNA than for DNA by approximately a factor of 2, but still sufficient for the measurement of µg amounts of RNA. The primary problem in attempting to determine the RNA content of crude cell extracts lies in distinguishing the contribution of RNA to the fluoresence signal from that of DNA.

KARSTEN and WOLLENBERGER (1972) showed that this aim can be achieved by measuring fluorescence before and after RNase digestion and taking the difference between the two measurements as the RNA signal. There are a number of complications which might lead to inaccurate estimation of the RNA signal: complexing with protein might hinder interaction of RNA with dye (as it does with DNA) or make a fraction of the RNA refractory to RNase digestion, and endogenous RNases might give rise to erroneously low estimates of the initial RNA content of an extract. All these sources of error would lead to an underestimate of the amount of RNA present. This method therefore provides a **minimum** estimate of RNA.

Reagents

see M.1.2.2 (p. 19)

Sample preparation

see M.1.2.2 (p. 19)

Assay Procedure

Fluorescence was measured from samples treated in a number of different ways. The composition of the individual treatments is given in Table 4.

	TREATMENT							
	A	B	C	D	E	F	Fluor-esence Blank (B_E)	Sample Blank (B_S)
Component	Amount of component added in µl							
Sample	100	100	100	100	100	100	As for A - F	As for A - F
Protein-ase K	-	-	-	-	100	100	but sam-ple re-placed	but eth-idium re-placed
RNase	-	-	-	100	-	100	by 100 µl DPBS	by 200 µl DPBS
DPBS	700	700	700	600	600	500		
Ethidium bromide	200	200	200	200	200	200		
Incubation 37 °C	No	No	Yes	Yes	Yes	Yes		
Time 37°C (min)	-	-	30	30	60	60		
Time Room Temp (hr)	-	6	<1	1	1	1.5		

Table 4 **Composition of sample treatments used for the fluorimetric determination of RNA using ethidium bromide. See text for explanation.**

Treatment F corresponds to that used for the estimation of DNA (cf. M.1.2.2.). Except for treatments A and B ethidium bromide was added subsequent to the 37°C

incubation after the samples had cooled. The rationale for this procedure was the desire to avoid effects which might have arisen either from temperature dependent dye-nucleic acid interactions or ethidium's inhibitory effect on enzyme activity (KARSTEN and WOLLENBERGER 1972).

Fluorescence measurement: as for DNA (see M.1.2.2). Fluoresence signals were corrected according to the formula

$$F_{TC} = F_T - (F_{BTE} + F_{BTS})$$

where T signifies the particular treatment

F_{TC} = corrected fluoresence
F_T = measured fluoresence
F_{BTE}, F_{BTS} = background blanks (see Table 4)

The following difference signals (which could all a priori be used as measures of RNA) were then obtained.

$F_{RNA(1)} = F_{AC} - F_{DC}$ (Untreated - 37°C RNase)

$F_{RNA(2)} = F_{CC} - F_{DC}$ (37°C - 37°C RNase)

$F_{RNA(3)} = F_{EC} - F_{FC}$ (37°C Proteinase -) (37°C Proteinase + RNase)

In addition $F_{AC} - F_{BC}$ and $F_{AC}-F_{CC}$ gave a rough measure of the extent of endogenous RNase activity.

The RNA fluoresence was calibrated against a set of standards made up from a stock solution of highly purified bacterial 5S RNA (Boehringer), whose concentration had been determined spectrophotometrically assuming $\varepsilon^{1\%}_{1cm} = 240$ at 260nm.

M.1.3.1 Limitations of this method

Apart from the general difficulties associated with using ethidium bromide (see beginning of sections M.1.2.2 and M.1.3), there is a further intrinsic limitation.

The method relies upon the measurement of differences between the fluorescence intensity of samples incubated with or without RNase. Each of these values is itself a difference value obtained after correction for a blank value; the blanks themselves might vary from treatment to treatment and this too must be determined. The errors in such difference determinations are cumulative. It follows that the sensitivity and accuracy of the method is going to be determined by the variation in the various contributory measurements.

Confidence in the accuracy of the method is going to be limited by the precision with which the variation parameter can be estimated, and this can only be achieved by providing an adequate amount of replication. The amount of material may often limit the number of times which a particular sample measurement can be repeated, but this restriction does not apply to the measurement of blank values, and as was noted above the readings from all RNA containing samples must be corrected for the appropriate blank. There is therefore much to be gained by making the estimates of blanks precise. This detail was neglected in the set of determinations described in Section R.9.1.1.6. This created a number of problems in ascertaining the accuracy of the estimates in samples containing small amounts of RNA. Although in this instance, it was possible to get round these difficulties, in general adequate (at least triplicate) replication should be used to minimise uncertainty in estimates of blank corrections.

M.1.4 Gel Electrophoresis

M.1.4.1 SDS Polyacrylamide gels

Analysis of the molecular weights of polypeptides

contained in protein preparations was performed using the method of SDS-polyacrylamide gel system of LAEMMLI (1970). Proteins were dissociated into their constituent polypeptide chains by boiling in mercaptoethanol and SDS. Where not otherwise stated gels containing 12.5% acrylamide, 0.3% bisacrylamide were used. In some cases gels containing a gradient of polyacrylamide concentration were used. These were made using the method of O'FARRELL (1975). A gradient of 10-17% was used unless otherwise stated.

Gels were 1 mm thick. Either 12 or 18 samples could be applied to each gel, depending on the comb used to form the application slots. The maximum volume which could be applied was 40 µl (12 slots) or 30 µl (18 slots). Most gels were electrophoresed at room temperature, but some later analyses were electrophoresed in the cold because this was found to improve the resolution of the separation.

For application to the gel samples were taken up in a mixture made up as follows:

Constituent	Parts by volume
SDS (20% aqueous solution)	20
87% Glycerin	20
Mercaptoethanol	10
1 M Tris pH 6.8	12.5
Bromophenol blue (0.1% aqueous solution)	2

At most 2 parts of the the sample to be analysed were mixed with at least 1 part of the application mixture and heated to 100°C for 5 minutes in a boiling water bath. In some analyses, in which it was necessary to analyse intact protein rather than individual polypeptide chains (see e.g. R.3.5.5 p.171), unreduced protein was electrophoresed after being taken up, without heating, in an application mixture from which mercaptoethanol had been omitted.

M.1.4.2 Isoelectric focusing

A number of different procedures were used. 1 mm gels were polymerised from a stock solution which always contained (w/v) 5% acrylamide and 0.2% bisacrylamide and in addition

either 4M or 6M Urea
or 3M Sucrose
or 10% Glycerol (approx. 10M).

In some earlier experiments the stock solution also contained 2% Triton X100.

No great difference in the focusing behaviour of marker proteins was noted using any of these different compositions. In most later experiments glycerol (COOPER 1977) was used for convenience.

To the stock solution was added a mixture of ampholines to give an ampholine composition of between 8% and 14%. The exact composition the ampholine mixture was varied according to the separation required and is given in the figure legends in RESULTS. TEMED was added to 0.1% and polymerisation initiated by addition of 0.1% ammonium persulphate. Gels were formed by pouring this solution into a 1 mm cassette to one surface of which the gel did not adhere (plexiglass or siliconised glass) and allowed to polymerise at room temperature for 2 hours. Before opening the cassettes were cooled to 4°C. The final concentration of acrylamide in the gels was between 4.3% (14% ampholine) and 4.6% (8% ampholine).

Focusing was carried out on a hollow metal plate through which a cooled water-alcohol mixture was circulated. The gels were brought into contact with liquid reservoirs, in which platinum electrodes were immersed, by strips of filter paper. The reservoir to which the cathode (+) was connected contained 100 mM phosphoric acid and the anodic (-) reservoir contained 200 mM sodium hydroxide (NaOH). In some experiments 1 M phosphoric acid and 1 M NaOH was used and the filter paper briges were soaked in a 2% ampholine solution (COOPER 1977),

but this did not lead to any noticeable improvement in gradient formation or focusing.

Gels were cooled to temperatures ranging between 0°C and 15°C. No difference in focusing performance was noted between these extremes, but irregularities (e.g. damage) in the gel which interfered with the conduction path caused less disturbance to the focusing pattern subsequently obtained when the higher temperature was used.

Gels were prefocused at 200V for 15 minutes. Samples (up to 10 µl) were applied directly to the surface of the gel using small flexible plastic rings. In earlier experiments gels were run overnight at a constant 500V and finally for 1 hour at 800V. In later experiments, after prefocusing, the gels were focused at either 8, 10 or 20W constant power (COOPER 1977). Under these conditions the potential across the gel rose from 300-600V to 1000V at which value it remained constant, because of limitations in the power supply, while the power and current applied to the gel fell.

After focusing, when measurement of the pH gradient in the gel was required, 1.0 cm strips were cut from each side of the gel and further cut up into 1 cm or 0.5 cm segments each of which was then eluted in 2 ml distilled water for at least 2 hours. The pH of the eluate was then read with a pH electrode.

Before staining the gels were fixed for 6 hours in the a solution made up as follows:

Methanol	300 ml
Distilled water	700 ml
Sulfosalycilic acid	34.5 g
Trichloacetic acid	115 g

This solution caused proteins, but not the ampholines, to precipitate. The ampholines could thus diffuse out of the gel, and did not interfere with the subsequent staining of proteins.

Focusing in very thin layer gels (0.1 mm) was performed according to GÖRG et al., (1981) using pH 2-11 ampholines, and

voltages up to 2000V.

Many of the marker proteins (e.g. horse and whale myoglobin) used to calibrate the gels (see legend to fig. 15 p. 178) give not one, but rather a complex set of bands upon focusing. This charge heterogeneity is often natural and does not necessarily represent impurities. Bands corresponding to published pI values were identified from the focusing patterns in the publication of ROSÉN and LINDSTRÖM (1979).

M.1.4.3 Staining of gels

M.1.4.3.1 Coomassie blue staining

Gels were stained for at least 2 hours in a solution of 0.1% Coomassie blue R250 (w/v), 50% Methanol (v/v) and 10% acetic (v/v) and destained in 16% acetic acid (v/v).

M.1.4.3.2 Silver staining

Coomassie blue stained gels (well destained) were poststained using TSANG et al's (1983) modification of the method of MERRIL et al. (1981).

M.2 Immunological Methods

M.2.1 Growth of Hybridoma Cells as Ascites Tumors in Mice

M.2.1.1 Mouse Husbandry

Balb/c (Balb/cHan derived from the Balb/cJ subline of Balb/c strains, ALTMAN and KATZ 1979) mice were used for ascites propagation. They were either obtained from the Zentralinstitut für Versuchstierzucht in Hannover, or were the progeny of a breeding colony established in Tübingen from

Hannover Stock. This colony was replaced early in 1983 with a colony of Balb/cByJ mice (derived from the /cAnN subline, ALTMAN and KATZ 1979) founded using breeding pairs obtained from the Jackson laboratories (U.S.A).

Mice for the production of ascites were either male or female and between 5 and 16 weeks old, except where otherwise noted. They were kept in either 7 litre cages (usually 2, at most 3 mice per cage) or 15 litre cages (up to 6 mice per cage) and fed ad libitum. The mice were kept in a well ventilated artificially-lit room under a 12 hour light-dark cycle.

It was essential to keep male mice older than 8 weeks at low stocking densities to minimise fighting. Older males sent from Hannover were often in a very poor state, as a result of fighting. They had matted fur, the tips of their tails were often bitten off or necrotic and their paws were often injured. They were moved to cages at lower stocking densities and allowed to recover for a week before being used.

Mice which accidently became injured as the result of handling e.g. when internal blood vessels ruptured as a result of intraperitoneal (i.p) injection were destroyed. Not only was this humane, but also prevented cannibalism (either because lightly injured mice became aggressive and attacked their cage mates or because very weak mice were killed by their healthy companions).

M.2.1.2 Intraperitoneal injection of Mice

Experienced animal handlers are able to inject live mice i.p.. I cannot. Mice were therefore lightly etherised before injection. Two deaths did occur as the direct result of etherisation, but this accounts for less than 1% of all mice handled. Mice were injected at a point slightly to the left of the midline, about 1/3 to 1/2 of the distance between the Anus and the Sternum, by inserting the the needle through the

abdominal wall at an oblique angle. The following needles were used:

	diam (mm)	length (mm)
for Pristan	0.65	32
for cell containing fluids:	1.10	30

The wide bore needles were TSK brand disposable ones with polished sharpened tips ("Lanzettenschliff") and it is important to use needles of this quality. Even when the peritoneum was swollen with ascites fluid und under pressure these needles could be inserted into the peritoneum without any loss of fluid, because the peritoneal membrane formed a seal around the needle. Needles of some other brands were so badly finished that they tended to tear the peritoneal membrane on entry preventing a seal from being formed.

M.2.1.3 Priming of Mice for Ascites Growth

Mice were injected i.p. with 0.3 ml Pristan (2,6,10,14 tetramethylpentadecane), and when required (see below) were irradiated with a 360 Rad whole body dose of gamma irradiation from a ^{137}Cs source made available to us by kind permission of Prof. Dr. J. KLEIN.

Mice could be kept for up to 10 days after Pristan priming and up to 4 days after irradiation, without any obvious detrimental effect on the growth of the ascites tumors. However when ascites were being established from cultured or frozen cells, the mice were prepared fresh. i.e. they were injected with cells immediately after irradiation and irradiation took place within 24 hours of Pristan priming.

M.2.1.4 Establishment of Ascites from Cell Culture

The steps described in this section and in section M.2.1.5 are only neccessary for establishing ascites from hybridomas derived from non-Balb/c mice.

At least 5 mice, between 7 and 14 weeks old were Pristan-primed and irradiated. Immediately after irradiation the mice were injected with between 5.10^6 and 10^7 hybridoma cells suspended in 0.4 ml DPBS. The hybridoma cells were collected from late log phase cultures (approx. 2.10^6 cell/ml), harvested by centrifugation (GLC1 Table Centrifuge, 700g, 5 min room temperature) and resuspended 10 fold concentrated in DPBS.

If ascites growth was not obtained within 12 weeks the mice were destroyed.

M.2.1.5 Propagation of Ascites and adaptation to growth in non-irradiated mice

The following scheme, described in detail below and outlined diagrammatically in fig. 2, represents a model selection strategy for obtaining and maintaining the growth of ascites in unirradiated mice. It is a composite construction derived from experience in establishing 15 different lines, and is judged to be the one most efficient in terms of human labour, number of mice and goal achievement.

Step 1 The first two ascites tumors to be established in the first passage are propagated further.

Step 2 Each of these ascites is injected into two irradiated and two unirradiated mice, to give a total of 4 sub-lines, A1' and B1' (unirradiated) and A2' and B2' (irradiated) in fig. 2, each comprising two mice.

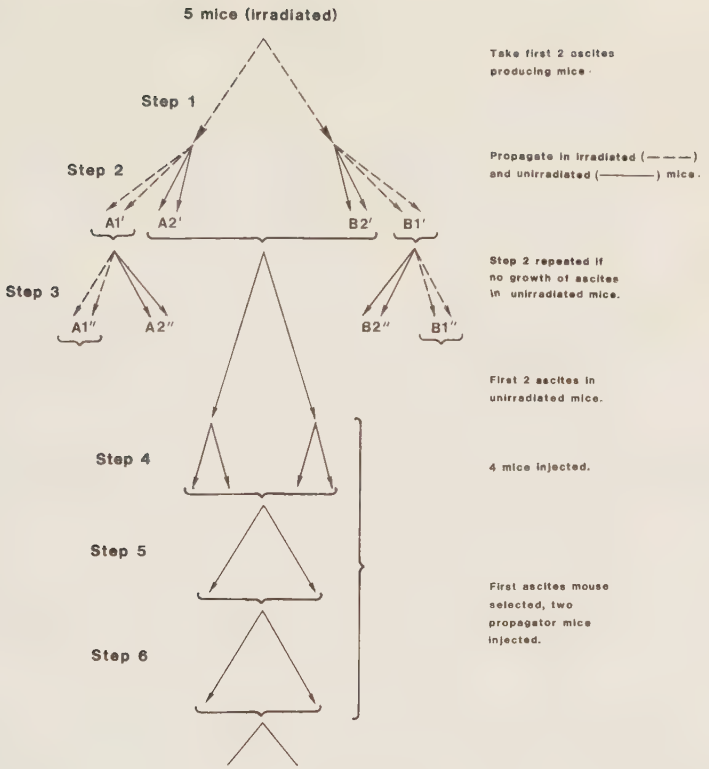

Fig. 2 **Strategy for establishing ascites of non Balb/c hybridomas in Balb/c mice.**
The arrows indicate the fate of individual mice. The designations A1,A2... refer to sublines. See text for full details of selection procedures.

Step 3 If no unirradiated mice had produced ascites then the first ascites obtained from each of the irradiated sublines was propagated further by repeating step 2 (lines A1'' and B1'' and A2'' and B2'' in fig. 2). Otherwise, until ascites growth was established in unirradiated mice, the following rule for the propagation of ascites in **irradiated** mice was

practised: if an unirradiated mouse either from the same passage or from a previous passage had produced ascites, then the ascites produced by the first irradiated mouse of a subline was injected into two further **irradiated** mice, and was maintained by repetition of this step until at least a second passage in unirradiated mice had been started. Thereafter propagation in irradiated mice ceased.

Step 4 Ascites from each of the first two unirradiated mice to produce tumors (regardless of subline) was propagated in two further unirradiated mice (second unirradiated passage: total 4 mice).

Step 5 The first ascites from the second unirradiated passage are injected into two further unirradiated mice (third unirradiated passage total 2 mice).

Step 6 The first ascites to be produced from the above pair is injected into a further unirradiated pair. The line is considered established.

Once step 6 has been reached, the number of mice in the passage can be increased, to allow the collection of large amounts of ascites fluid, or the line can be closed down after freezing down cells for later revival.

This scheme requires a maximum of 8 mice per passage in the early stages, and ensures continuous selection for the fastest growing cells.

The amount of ascites which was injected further at each passage was usually 0.3 ml. For very fast growing lines (passage time less than 1 week) this was reduced to 0.1 ml.

Occasionally solid tumors were obtained, especially during the early passages. These were minced with scissors and the fragments suspended in DPBS by gentle passage through a wide bore needle to allow transfer to other mice.

M.2.1.6 Harvest of Ascites and freezing of Cells

The mice were killed by breaking their necks. They were then pinned out on a dissection board and dowsed with methanol to wet and sterilise their fur. The abdominal skin was cut away to expose the semi-transparent peritoneal membrane, and a needle with a syringe attached was inserted through the membrane. The ascites fluid was then drawn off into the syringe. By observing the needle through the membrane it was possible to ensure that internal organs (e.g. the bladder) were not punctured and to prevent the tip of the needle from becoming blocked by loose tissue fragments. When further passaging was neccessary an aliquot of the harvested fluid was immediately injected into appropriately primed mice. After harvesting as much fluid as possible it was transferred to a sterile tube and the peritoneal membrane cut open to expose the abdominal cavity. The intestines were raised to allow access of the syringe to the dorsal abdominal wall, and any remaining fluid which had drained there was also collected.

The ascites fluid was centrifuged for 5 min at 2000 rpm in a GLC1 table centrifuge at room temperature. Any pristan present formed a transparent oily layer at the top of the tube and was carefully removed. The clear, slightly yellowish, aqueous supernatant, which contained the antibody, was then carefully separated from the pellet of cells and stored frozen at -70^{o}C, after taking a small sample to test for antibody activity. Occasionally a gelatinous mass formed when the ascites fluid was centrifuged which prevented separation of the cells from the supernatant. This problem could be overcome by simply shearing the gel (pipetting it up and down 2 or 3 times in a pasteur pipette) and recentrifuging.

When cells were required for freezing, the pellet was taken up in an arbitrary volume of RPMI culture medium, and aliquots containing an amount equivalent to 0.5ml of the original ascites were transferred to thick-walled 2 ml polypropylene sample tubes. (Greiner Cat Nr. 121261). DMSO to give

a final concentration of 10% v/v was then added and the tubes mixed rapidly by inversion. The tubes were then wrapped in tissue paper, placed in a styropor container and slowly frozen by placing them in a -70°C freezer. Frozen cells were stored in liquid nitrogen within one week of freezing.

M.2.1.7 Thawing of Cells

Tubes containing frozen cells were transferred to the laboratory on dry ice and then rapidly thawed by plunging into a 37° water bath and agitating slightly. Thawing took under 30 seconds using this procedure. The cells were centrifuged (GLC 1 Centrifuge 700g 5 min room temperature), and as much of the DMSO containing supernatant as possible was removed. The cells were resuspended in 0.4 ml DPBS and injected into freshly primed mice within 5 minutes of resuspension.

M.2.1.8 Testing Ascites Supernatants for Antibody Activity

The ascites supernatants were diluted 1 to 40 and tested for indirect immunofluoresent staining on either chromosome squashes (see M.2.6.1) or on fixed Kc cells (see M.2.6.2), depending on availability.

M.2.2 Other forms of antibody production

M.2.2.1 Hybridoma supernatants

see SAUMWEBER et al. (1980) and SAUMWEBER (1980) for details of *in vitro* growth of hybridomas.

M.2.2.2 Radioactively labelled monoclonal antibodies

Log phase hybridoma cultures (approx 10^6 cells/ml) were harvested by centrifugation (700 rpm GLC-1 centrifuge, 5 min room temperature) resuspended in DPBS at 10^7 cells/ml and incubated for 15 min at 37°C, centrifuged and resuspended in DMEM lacking lysine at 10^7 cells/ml. ^{14}C-lysine (uniformly labelled, Amersham 10 mCi/mmol, 37MBq/mmol) was added at a concentration of 2 µCi/ml (74 KBq/ml) and the cultures incubated for at least 12 hours (overnight) in the wells of microtiter plates under standard cell culture conditions. The medium usually turned bright yellow under these conditions. The plates were centrifuged (GLC-1 centrifuge with plate adapter, 700 rpm) and the antibody-containing supernatants carefully withdrawn with a pasteur pipette. A small amount of residual debris was removed by centifugation in an Eppendorf table centrifuge (8000 rpm, 2 minutes) and the supernatants stored frozen. Under these conditions the presence or absence of serum in the medium had negligible effect on the incorporation of precursor into the antibody. The ^{14}C lysine solution added to the cells contained a small amount of ethanol (final concentration 0.2%), but since in control experiments (not shown) ethanol at a concentrations 1% had no effect upon cell growth (rate of cell division), no attempt was made to remove it.

M.2.2.3 Rabbit Antisera against Calf Thymus Histone 1

Antisera with the designations 401-409 were produced by M.RENZ and P.NEHLS who kindly donated it to me. Antisera with designations 138-150 were produced in outbred rabbits obtained from a local rabbit dealer using a variety of immunisation methods (site of injection, type of adjuvant and emulsion etc.). Details of immunisation schedules for individual rabbits are presented under "results". The following materials

and techniques were employed. Antigen was always injected using either complete or incomplete Freund's adjuvant. Single and double emulsions of antigen and adjuvant were prepared using the "double hub syringe" technique described by HERBERT (1978). Intramuscular (i.m.) and subcutaneous (s.c.) were made at 5 or 6 different positions on the body. Intravenous (i.v) injections were made in the ear vein. Total volumes injected were 2 ml (single emulsion), 6 ml (double emulsion) and 1 ml (i.v.). The histone 1 used as antigen in these immunisations was kindly provided by Dr. M. Renz and was repurified before use as described in section M.2.3.1.2. Comercially obtained tRNA (Boehringer) was used to prepare complexes with H1 in the ratio 2 parts H1: 1part tRNA. All H1 and H1-tRNA complexes were made up in 0.85% NaCl. Single emulsions were made using H1 or H1-tRNA complexes containing 2% Tween 80.

After s.c. and i.m. immunisations bleedings were first made after two weeks, following i.v. immunisations after seven days. With the exception of the immunisations prior to the final bleeding the minimum time between immunisations was 3 weeks but was often longer. The most productive rabbits were immunised 14 times within a period of 18 months. Rabbits were bled from the ear vein and between 20 and 40 ml blood were collected at each bleeding. For the final bleeding 2 immunisations with 400 µg H1 were made 7 days apart: the first s/c using complete Freund's adjuvant, the second i.v. in incomplete Freund's adjuvant. The final bleeding was made from the jugular vein and yielded between 80 and 100 ml. The blood was allowed to coagulate at 37°C for 1 hour. After standing overnight on ice serum was decanted from the clot and stored at -70°C until use.

M.2.3. Antibody Purification

M.2.3.1 From Rabbit Serum

M.2.3.1.1 IgG Purification

Sera were clarified by centrifugation in a SORVALL HB4 rotor at 13,000 rpm for 15 min ($0^{\circ}C$), followed by sequential filtration through 0.45 and 0.22 μ Millipore filters. 0.75 volumes of saturated ammonium sulfate (room temperature) was dripped into the clarified sera over a period of 4 hours while stirring on ice to make a 43% saturated solution and the resulting suspension stirred for a further 12 hours (overnight). All subsequent steps were carried out in the cold (cold room $4^{\circ}C$-$8^{\circ}C$). The precipitate was collected by centrifuging at 8000 rpm in an HB4 rotor for 20 min ($0^{\circ}C$), resuspended in 1.5 times the initial volume of 50% saturated (room temperature) ammonium sulphate and the precipitate again collected. Much coloured material (haemoglobin in sera derived from haemolysed blood) was removed in the 43-50% AS wash step. The precipitate was dissolved in approx. 1/5 the original volume of 0.85% NaCl and dialysed extensively against the appropriate buffer for the next purification step.

IgG was purified by ion exchange chromatography on DEAE-cellulose. DEAE cellulose DE52 (Whatman) was prepared according to the manufacturers instructions by removing fines and treating with acid and alkali. It was then equilibrated against 0.0175 M $NaPO_4$ buffer pH 6.3. The IgG containing AS fraction was dialysed against the same buffer and applied to the column. The flow through fractions (often slightly retarded) were collected, extensively dialysed against distilled water and lyophilised.

The IgG from one serum was purified directly by chromatography on "DEAE affi-gel-blue", a polyacrylamide matrix, in bead form, derivatised with DEAE groups and Cibacron blue (produced by the firm BIORAD). Serum albumin binds tightly to

Cibacron blue and most other serum proteins absorb to the DEAE groups, leaving IgG in the flow-through. It was prepared according to the manufacturers instructions and equilibrated with 0.02M Tris-Cl, 0.028M NaCl, 0.02% NaN_3, pH 8.0. (DAGB buffer). Antiserum was dialysed against the same buffer and applied to the column. The antiserum was washed onto the column with DAGB buffer and the flow-through and fractions eluted with 0.5M and 1.4M NaCl in DAGB buffer were collected and assayed for specific antibody content.

M.2.3.1.2 Affinity Chromatography on Calf-Thymus H1-Sepharose

The Histone 1 used in these preparations was kindly provided by Dr. M.Renz. This material was purified by gel-exclusion chromatography on Sepharose 6B-Cl to remove a low M.W. contaminant of an unknown nature which absorbed at 230 nm but was not identifiable after SDS-PAGE electrophoresis.

Derivatisation of Sepharose was performed as follows. All steps were carried out at room temperature except where noted. 2 grams of cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) was rinsed with 400 ml 1mM HCl on a glass filter (Schott 17G3) over a period of 10 to 15 min and then suspended in 0.1M $NaHCO_3$, 0.2M NaCl and shaken on a water bath at 25°C for 3hr. This step was designed to deactivate the matrix and prevent too much multipoint attachment of the highly lysine-rich H1. 15 mg H1 was added and the reaction mixture shaken for a further 2.5 hr. The derivatised Sepharose was placed on a glass filter and washed with 50 ml 1mM HCl, 0.4M NaCl (approx. 30% of the H1 was removed with this step). The material was then incubated for 1 hr at 25°C with 1M ethanolamine, 10mM Tris-Cl pH 8.5 to block any unreacted groups on the Sepharose, washed 6 times alternately with 50 ml 0.1mM HCl, 0.4M NaCl and 50 ml 10mM Tris-Cl pH 8.5, 0.4M NaCl, with no further detectable loss of $O.D._{230}$ absorbing material, poured into a column and washed overnight with PBS. The column was transferred to the cold and all subsequent steps were

carried out in the cold. Before it was first used the material was eluted with 4M $MgCl_2$ (the highest salt concentration to which it would be exposed during affinity chromatography), and an amount of $O.D._{230}$ absorbing material equivalent to 2 mg H1 was loosed from the column by this procedure. The volume of the column was 7ml and was derivatised with approx. 1 mg H1 per ml.

The AS fraction, or DEAE purified IgG, to be affinity purified (10mg protein/ml in PBS) was made 1 mM in PMSF by addition of 1/200 volume of a freshly made 0.2M solution in ethanol and pumped over the column. The addition of PMSF was designed to protect H1 from proteolytic attack. The column was then washed with PBS until the $O.D._{280}$ of the eluate was the same as that of the input buffer. Strongly bound antibody was eluted from the column by applying sequentially 1M NaCl, 2.5M $MgCl_2$ and 4M $MgCl_2$ each made up in PBS. Immediately after elution, protein containing fractions were pooled and dialysed against several changes of PBS. After concentration in an Amicon cell, using an XM50 filter, protein concentration was measured, affinity purified antibody divided into aliquots and stored frozen at -70°C.

M.2.3.2 From mouse ascites fluid

The purification schemes outlined in table 5 were used.

Step 1 Clarification

The ascites fluid from several mice was thawed overnight at 4°C and pooled to give a volume between 50 and 100 ml. The pooled material was often slightly turbid and was clarified by centrifugation (15 min 4°C at 7,500 rpm in the HB4 rotor of a Sorvall RC-5 centrifuge). The ascites fluid formed a clear yellow aqueous phase and was carefully removed from the tube with a pipette. Care was taken not to disturb either the thin upper layer of oil, or the small pellet made up of tissue

debris. Clarification by filtration 0.8 μ pore size millipore filters was also attempted for some ascites but the filters clogged too rapidly for this technique to be useful.

Step Number	Antibody (sub-)Class	Procedure
1	all	Clarification
2	all	Ammonium sulphate precipitation
3	IgG	Gel exclusion chromatography on Sephacryl S300 column
	IgM	Affinity chromatography on Con A sepharose
4	IgG1	Chromatofocussing/Protein A sepharose pH 6.0 eluate
	IgG2	Affinity chromatography on Protein A sepharose
	IgM	(Gel exclusion chromatography on Sephacryl S300 column) - Optional

Table 5 **Purification scheme for Antibodies in Ascites fluid**

- -

M.2.3.2.1 Preparation of IgG-enriched fraction

Ammonium Sulphate (AS) Precipitation

Unless otherwise stated

i) all procedures were carried out at 0°C (water/ice bath or refrigerated centrifuge).

ii) centrifugation was for 15 min at 7,500 rpm in the HB4 rotor of a Sorvall RC-5 Centrifuge.

iii) supernatants from centrifugation steps were removed by decanting

iv) all additions were mixed by magnetic stirring

v) all "% saturation" values refer to the amount of AS in

a solution which is saturated at room temperature (20°C).

Two intermediate stages, a "60% AS pellet" and a "25% AS supernatant", preceded production of the final pellet, a 25%-60% "cut" of the original material.

60% Pellet

A saturated solution of AS (1.5x the volume of clarified ascites) was slowly dripped into the clarified ascites over a period of approx. 2 hr., to give a 60% saturated solution. The turbid suspension was stirred for at least a further 4 hours, usually overnight, and centrifuged. A solid white film (?low density lipoproteins salted out of solution?) often formed on the surface of the supernatant and was not removed by decantation. It was eliminated by wiping the inside of the centrifuge tube with a paper tissue.

25% Supernatant

The 60% pellet was suspended in a volume of 25% saturated AS equal to that of the original ascites, stirred for 3 hours, centrifuged and the small pellet discarded.

25-60% "cut"

Solid AS at a rate of 22.6g/100ml of 25% supernatant was added in the form of 6 portions, shaken in from the tip of a spatula at 15 minute intervals. The suspension was stirred for a further four hours and centrifuged. The white pellet was either stored frozen at -20°C or dissolved in a minimum volume of PBS (usually between 1/5 and 1/10 the original volume of the clarified ascites, depending on the solubility of the particular antibody) and further processed immediately.

Modified Method

Because of solubility problems associated with a small proportion of the antibodies (R.3.4.3), and in order to retain a standard procedure, from mid-1983 onwards the 25%

supernatant and 25-60% cut steps have been omitted from the purification procedure. The 60% pellet is now taken up in a minimum volume of PBS and applied directly to a Sephacryl column (next step).

Sephacryl S300 Gel Exclusion Chromatography

Sephacryl S300 was prepared according to the manufacturers' instructions, and packed in columns whose length/diameter ratio exceeded 20. Different sized columns were used for different preparations. The largest preparations were processed in a 90 x 4 cm column which contained 1.2 l of Sephacryl. The column was washed with at least 3 column volumes of PBS between separations. The material to be separated was ideally applied in a volume which was less than 1% of the column volume and eluted at a rate of about 1 column volume per 24 hours. Elution was followed spctrophotometrically at 280 nm using a Gilson flow through machine attached to an LKB chart recorder. When the 1.2 l column was used 10 ml fractions were collected. Fractions containing antibody, and separated from other proteins, were pooled and dialysed against the appropriate buffer for the separation outlined in step 4. Chromatography was carried out in a cold room (4-8^{o}C). When not in use the column contained 0.1% Sodium azide in PBS as preservative. At periods of about 6 months the column was "cleansed" by running one column volume of a 1% solution of SDS through the column at room temperature, followed by several column volumes of distilled water also at room temperature, and then reequilibrated with PBS in the cold.

M.2.3.3.2 Preparation of IgM containing Fraction

The AS "cut" fraction was produced as in section M.2.3.3.1 and taken up in a volume of 0.5M NaCl in PBS 1/5 that of the original ascites. High salt had previously been

found useful for keeping mouse IgM in solution at high concentrations (H. SINGER personal communication). This material was subjected directly to affinity chromatography.

M.2.3.3.3 Affinity Chromatography of IgM on Con A sepharose

The ConA Sepharose(PHARMACIA) was prepared according to the manufacturers' instructions and equilibrated in 0.5M NaCl, 20 mM Tris-Cl pH 7.4, (M buffer). For 2ml of ascites a 3 ml column was used. The IgM containing fraction described in the previous section was applied to the column and the column was then washed with M buffer until unbound material had been washed out. Low affinity conA binding proteins were eluted with several column volumes of 50mM α-methyl mannoside in M buffer and IgM was then eluted with 0.5M α-methyl-mannoside in the same buffer. Fractions containing IgM were pooled, precipitated with 2 volumes saturated ammonium sulphate, taken up in a small volume of PBS, dialysed against the same buffer to remove any remaining traces of α-methyl mannoside and stored frozen at -70°C. In one experiment this material was further chromatographed over a small Sephacryl S300 (2.6 x 60 cm - 190 ml) column equilibrated in PBS, to separate it from putative glycoprotein contaminants but the IgM was already essentially pure by this stage (see R.3.3.1). The IgM containing fractions were pooled and stored frozen at -70°C.

M.2.3.3.4 Affinity Chromatography of IgG subclasses on Protein A Sepharose

Protein A Sepharose(Pharmacia) was prepared according to the manufacturers' instructions. For purification of IgG2a or IgG3 S300 fractions were dialysed against citrate buffer pH 6.0, and applied to a Protein A column equilibrated with this buffer. After applying the S300 fraction the column was washed in the same buffer. Protein in the eluate was monitored

spectrophotometrically at 280nm with a Gilson flow-through spectrophotometer. When the optical absorbtion of the eluted material had returned to that of the buffer, which often required a considerable amount of washing because of retarded material, IgG2a was eluted by applying Citrate buffer pH 4.5 to the column. The eluted fractions were collected in tubes containing sufficient 1.0M Tris to neutralise the citrate in the eluate. It would also be possible to purify IgG2b, uncontaminated by other subclasses, by eluting first with pH 4.5 (to remove IgG2a and IgG3) and then collecting material eluted with pH 3.0 citrate buffer.

Some IgG1 antibodies could be applied in pH 6.0 buffer and recovered as retarded material in the pH 6 eluant, but the degree of retention and thus the separation from flow-through material varied from antibody to antibody and was never entirely satisfactory. An number of alternative schemes for purification of IgG1 were attempted based on the method of DRESSER (1978) which depended on retention of this subclass by protein A in 10mM Tris-Cl pH 8.1 and its release by either either pH 5.7 or pH 6.5 buffer. In later preparations IgG1 containing fractions in 100 mM Tris-citrate buffer pH 8.5 were applied to protein A equilibrated in the same buffer and eluted with citrate buffer pH 5.7. An important limitation to this method is that the IgG1 must be applied in less than 1 column volume and that not more than 1 mg IgG1/ml packed column volume should be applied. The column was regenerated for further use by elution with 2 column volumes of pH 3.0 citrate buffer and stored in PBS containing 0.1% azide.

Eluted fractions containing the purified subfractions were usually concentrated by AS precipitation, redissolved in PBS, dialysed against 1/10 diluted PBS, divided up into aliquots and stored frozen at -70^{o}C.

M.2.3.3.5 Chromatofocussing

40ml of the weak ion exchanger PBE 94 (PHARMACIA) was prepared according to the manufacturers' instructions and poured into a 1cm diam. x 60cm column and allowed to settle under gravity (bed height 51cm). After overlayering the column with a 2cm layer of Sephadex G50 coarse the column was equilibrated with 500 ml start buffer (see table 6). The IgG enriched fractions which had been dialysed either against distilled water or 1/10 diluted PBS (PBS/10) were concentrated to a volume of 10 ml (25% of the column volume) using an Amicon ultrafiltration cell with a Berghof BM500 membrane and adjusted to start buffer concentration and pH by addition of concentrated start buffer. 2-3ml of elution buffer (a mixture of Polybuffers 96 and 74) adjusted to the pH shown in table 6 was applied to the column and then the antibody containing solution.

Hybridoma	Protein Concentration applied to column. (mg/ml)	Start Buffer pH	Elution Buffer pH
P12/8/6	3.6	7.4[1]	4.5[1]
Z4/10/9	8.4*	7.0[1]	3.9[1]
T7/3/3/4	5.3	7.4	3.9
X4/8/6	3.0	7.4	4.0
S5/7/7/6	n.d	7.4/6.2+	4.3

+ Two different preparations. * probably incorrect: light scattering due to incipient precipitation n.d. not determined
[1] Imidazole acetate and polybuffer acetate were used for these preparations. The manufacturers instruction booklet contains contradictory information about the use of acetate. Its use is both recommended and warned against. Chloride was used as anion for the other preparations.

Table 6 **Start and elution buffer conditions for chromatofocussing of IgG1 antibodies.**

Bound protein was eluted with 14 volumes of elution buffer allowed to flow through the column at a flow rate of 12.5 ml hr^{-1} under hydrostatic pressure. 5 ml fractions were

collected. Chromatography was carried out in a cold room (4-10°C). $O.D_{280}$ in the eluate was monitored with a Gilson flow-through spectrophotometer. Fractions containing antibody were pooled and antibody precipitated by addition of 1.5 volumes of saturated AS (for details see step 1, M.2.3.2.1, p. 42). Following centrifugation, most of the polybuffer was removed in the supernatant. Remaining traces of Polybuffer and AS were removed by passage through a Sephacryl S200 column containing 1M NaCl in PBS. The IgG peak was dialysed against PBS or PBS/10 and stored frozen at -70°C.

pH in the eluate was measured after allowing the fractions to equilibrate with air at room temperature; pH in the antibody containing fractions was usually not measured directly but was estimated by interpolation. The column was regenerated by washing off strongly absorbed proteins with 1M NaCl in start buffer before further use.

M.2.4 Antibody Derivatisation

M.2.4.1 Fluorochrome labelling

M.2.4.1.1 Fluorescein Isothiocyanate (FITC)

To 400 µl of purified antibody at a concentration of approximately 1 mg/ml was added 100 µl of 1 mg/ml FITC dissolved in 0.5 M Na_2CO_3, 0.15 M NaCl pH 9.5. The reaction mixture was incubated for 1.5 hr at room temperature with constant mixing (rotation). Free FITC was separated from labelled protein by chromatography over a 7.5 ml Sephadex G25 (Sephadex G50 can also be used) column equilibrated with PBS. Coloured material eluting in the void volume was pooled and the $O.D._{280}$ and $O.D._{495}$ was measured. The molar ratio of fluorochrome to IgG was calculated according to the formula:

$$\text{mole FITC/mole IgG} = \frac{2.79 \times O.D._{495}}{O.D._{280} - (0.35 \times O.D._{495})}$$

modified from THE and FELTKAMP (1970) to take into account the decrease in absorbance at 495 nm by a factor of 1.13 that FITC undergoes upon conjugation to protein (DANDLIKER et al., 1964, McKINNEY et al., 1966, TENGERDY and CHANG 1966, STEINBACH 1974, STEINBACH and von MAYERSBACH 1976).

The amount of protein in the protein conjugate can be estimated from the $O.D._{280}$ corrected for adsorption by fluorochrome at this wavelength (bottom line of the right hand side of the above equation). The correction factor of 0.35 is derived from an estimate by WOOD et al., (1965) using an FITC conjugate of gelatin.

The above estimate is based upon a molar extinction coefficient of $8.5\ 10^4$ at 495 nm for free FITC (McKINNEY et al., 1964). There is a discrepant report of a lower extinction coefficient and a larger decrease in FITC absorbance following conjugation which would give 60% greater estimates of the degree of substitution if it were correct (JOBBÁGY and JOBBÁGY 1972a,b). However this report is almost certainly in error: the authors have used a correction for light scattering ("irrelevant absorption") which is incorrect. When this "correction" is omitted, their data is in agreement with that of the other investigators.

Nonetheless, it should be pointed out that the molar extinction coefficient which has been used above is only valid for the pH interval 8.0 to 12.0 under which conditions it is invariant. Below pH 8 the optical absorption of FITC (both free and protein-bound) at 495 nm decreases. The exact amount of the decrease and its dependence upon pH for protein conjugates varies somewhat from protein to protein (STEINBACH and VON MEYERSBACH 1976). Use of the formula given above to calculate the extent of FITC substitution will lead to underestimates of about 20% if absorption measurements are made at pH 7.0.

The distribution of conjugates bearing 0,1,2.....n

fluorochrome ligands can be calculated from the binomial distribution:

$$P(x)_o^n = C \left[\frac{F/P}{n}\right]^x \left[1 - \frac{F/P}{n}\right]^{(n-x)}$$

where $P(x)_o^n$ = fractional probability of obtaining by chance a molecule substituted x times from a population containing molecules substituted from 0 to n times.
x = number of residues substituted
C = binomial coefficient for x from n
F/P = mean degree of labelling (mol/mol)
and n = maximum number of fluorochrome ligands which can bind per IgG molecule (should be found experimentally, but n=8 should be a reasonable approximation).

(BLAKESLEE and BAINES 1976).

FITC-IgG conjugates were stored on ice in the dark (aluminium foil wrapped tubes).

M.2.4.1.2 TetramethylRhodamine Isothiocyanate (TRITC)

Crystalline TRITC from the Baltimore Biological Laboratories (a subsiduary of Becton-Dickinson) was used. To 400 µl of purified antibody at a concentration of approximately 1 mg/ml was added 50 µl 0.5 M Na_2CO_3, 0.15 M NaCl pH 9.5. A 4 mg/ml solution of TRITC in DMSO was then added at the rate of 25 µg TRITC/mg protein. The reaction mixture was incubated for 3 hours in the cold with constant mixing (rotation). Free dye was separated from the protein dye conjugate by Sephadex chromatography as for FITC (see above). In some early experiments the reaction was carried out at room temperature for three hours, but did not prove successful (cf. R.3.4.4).

The molar ratio of TRITC to IgG was calculated according to the formula:

$$\text{Mole TRITC/Mole IgG} = \frac{4.34 \times O.D._{555}}{O.D._{280} - (0.64 \times O.D._{555})}$$

The factor 4.34 is based on the ratio between the absorption coefficients of conjugates of FITC and TRITC, at 495 nm and 555 nm respectively, obtained by VAN DALEN and HAAIJMAN (1974). These values were measured at pH 7.8. The pH dependence (or independence) of conjugated TRITC has not been investigated.

There are some discrepancies between my results using crystalline TRITC and those reported in the literature. AMANTE et al., (1972) have reported that "amorphous" and "crystalline" TRITC vary in their spectral characteristics. However the crystalline TRITC which I obtained came from the same source as their amorphous TRITC and had very similar spectral characteristics. My estimate of the $O.D._{555}/O.D._{515}$ ratio of the free fluorochrome was 1.15 compared to their value of 1.2, and in the conjugate my estimate of $O.D._{555}/O.D._{515}$ is 0.89 compared to their value of 0.91. In contrast the $O.D._{555}/O.D._{515}$ values they reported for the free and bound forms of their "crystalline" TRITC were 2.0 and 1.4 respectively.

AMANTE et al. (1972) claim to have found no absorbtion of conjugated crystalline TRITC at $O.D._{280}$, which seems unlikely. On the premise that this observation was in error, and since the $O.D._{280}$ contribution from the fluorochrome is required to estimate both the amount of antibody in the conjugate, as well as the fluorochrome/protein ratio, I determined its value (as a proportion of $O.D._{555}$) by measuring the spectra of myoglobin and myoglobin-TRITC. The absorption values of the spectra could be normalised with respect to the major Soret band at 408 nm (AWAD et al., 1963). The relative contribution of bound fluorochrome at this wavelength is negligible (<1% of the Soret absorbance). The spectrum of conjugated TRITC can then derived from the (calculated) difference between the two spectra. The estimate of $O.D._{280}/O.D._{555}$ which I obtain from the difference spectrum is 0.64 which is very similar to the

value of 0.62 obtained by CEBRA and GOLDSTEIN (1965) using a TRITC-gelatine conjugate (I have calculated this value by multiplying their published value by a factor 1/0.9 assuming that their conjugated TRITC has the spectral characteristics of AMANTE et al.'s "amorphous TRITC" - see above. My direct measurement of $O.D._{280}/O.D._{515}$ of 0.59 was close to their value of 0.56).

There appears to be very little difference between the behaviour of the crystalline TRITC which is currently commercially available and the "amorphous" substance characterised by AMANTE et al. (1972) and suggests that the "crystalline" material which they examined may not have been TRITC. Presumably the manufacture of TRITC is now standardised. Thus, contrary to the opinion of FORNI (1979), it does appear possible to estimate both the flurochrome/protein ratio and the true amount of protein in TRITC conjugates.

The TRITC conjugates were stored on ice in the dark (aluminium foil wrapped tubes). However, even under these conditions the conjugate may be unstable. After 4 days storage the absorbance of a TRITC-Myoglobin conjugate at both 555 nm and of the TRITC-derived absorbance at 280 nm decreased by 20% with respect to absorption in the 408 nm Soret band. It is therefore advisable to use such conjugates within a short time of production. A single preparation was frozen, but upon thawing much of the conjugate was found to be precipitated, so no further attempt was made to find out whether freezing would increase the stability of the conjugates.

M.2.4.2 Iodination

RAM-Fab was labelled as described in RISAU et al. (1981), with the modification of using Sephadex G75 rather than G50 to separate unreacted iodine from labelled protein.

M.2.4.3 Production of Fab fragments

Two different methods were used for the production of Fab fragments. One batch of P11/6/3 Fab was produced by trypsin digestion, molecular sieve separation of the digested products, and removal of residual contaminating Fc fragments by Protein A chromatography either as described in SAUMWEBER (1980). This method worked but caused the formation of a large precipitate which was difficult to handle. For this reason an alternative method based on procedures described by PARHAM et al., (1982) was employed.

The digestion procedure described below was designed primarily to test the sensitivity of different antibodies to trypsin and only sufficient protein was processed to satisfy analytical requirements. The reactions were carried out without stirring because of the small volumes involved. For preparative digestions, in larger volumes, stirring would probably be required and the time course of the reaction might change, so that the precise amounts of reagents might have to adjusted slightly.

100 µl purified monoclonal antibody in PBS was diluted to about 2.5 mg/ml with PBS and adjusted to 100 mM Tris pH 7.5, 10 mM cysteine and 2 mM EDTA (digestion buffer) with a 10 fold concentrated stock solution. It was essential to make up the concentrated stock solution fresh as the cysteine precipitated within 24 hours at room temperature, presumably as a consequence of oxidation to cystine. All buffers were degassed before use. Papain (Serva 38 mg/ml, 10 U/ml) was diluted 1/190 in digestion buffer and added to antibody at a rate of 1 µg papain/100 µg antibody. After thorough mixing the digestion was allowed to proceed at 37°C for an appropriate length of time (varies for different antibodies see R.3.4.5, the range for optimal digestion under these conditions lies between 30 min and >4 hr) and was stopped addition of iodoacetamide to a final concentration of 11 mM. Control experiments in which iodoacetamide was added at the beginning of the incubation

period showed that this amount of iodoacetamide completely inhibited the enzyme reaction. The inhibited digest could be used directly for ELISA.

The specific activity of commercially available papain with the same nominal specific activity varies between suppliers and between different batches from the same supplier (R. HALLMANN pers. comm.). It is therefore essential to carry out preliminary experiments to determine the time course of digestion for a particular batch of enzyme before attempting any large scale production of Fab fragments. If the time course of the reaction is inconveniently fast or slow it may be necessary to alter the amount of enzyme from that which I used.

M.2.5 Assay methods for Antibodies and Antigens

M.2.5.1 General Methods

M.2.5.1.1 Ouchterlony Double Immunodiffusion (qualitative and quantitative)

The principle of this method is described in OUCHTERLONY and NILSSON (1978). We used microimmunodiffusion plates made on microscope slides. The Agar had the following composition.

	g/l
Agar	15
NaCl	7.0
Na_3citrate.$2H_2O$	4.95
NaN_3	0.3
PEG 1000	25

A large volume of stock solution was made and could be stored in 200 ml bottles for up to 6 months in the cold. The stock was melted in a boiling water bath allowed to cool and kept in a water bath at 40°C for pouring. Cleaned microscope slides were each carefully layered with 3ml of the stock solution using a pipette. The agar was held on the slide by

surface tension until it gelled. 3 sets of double immunodiffusion wells, comprising a large central well with a volume of approx. 25 µl surrounded by 6 equidistant outer wells each with a volume of approx. 5 µl, were then stamped out in the agar using a template. The plates were stored in the cold under conditions of high humidity.

Antigen or antibody could be placed in the central well depending on the property to be assayed. Antibody or antigen respectively was then placed in the peripheral wells and immunoprecipitation lines allowed to develop for at least 12 hours (overnight) at room temperature. Immunoprecipitation lines with some high M.W antigens (e.g. IgM) sometimes took up to 18 hrs to develop fully. For reference purposes developed plates could be stored in the cold for several days, or photographed against diffuse background illumination.

The method could be made semiquantitative by titrating known amounts of antibody or antigen against its complement in factor two dilution steps and determining the concentration at which precipitation lines could no longer be detected. Comparisons between the titrations of an unknown and those of a standard allow estimates of the amount of unknown within a factor 2-4. The standard and the unknown must be titrated at the same time, because the plates age and the absolute concentration at which the end point occurs will vary with time.

M.2.5.1.2 Complement Fixation

M.2.5.1.2.1 Haemoglobin Release

The quantitative micro-complement fixation method of WASSERMAN and LEVINE (1961) was used.

Preparation of sensitised sheep erythrocytes

Sheep erythrocytes were obtained from a sheep belonging to the MPI für Virusforschung and stored in the cold in Alsever's solution (0.138 M glucose, 0.027M Na_3citrate, 0.072M

NaCl, adjusted to pH 6.1 with 0.1M citric acid, 100 U/ml Penicillin, 50 µg/ml streptomycin) for up to 5 weeks. Before use the cells were washed 3 times by centrifugation (700 rpm GLC-1 centrifuge) and gentle resuspension in a large volume of 0.85% w/v ice-cold NaCl (0.146 M). After the final wash the cells were resuspended in KBR buffer (0.0027M 5,5 diethylbarituric acid, 0.0018M Na diethylbarbiturate, 0.143M NaCl, 0.003M $NaHCO_3$, 4.9 10^{-4}M $MgCl_2$, 1.36 10^{-4}M $CaCl_2$, 0.1% BSA) and counted. The cell concentration was adjusted to 5.10^8 cells/ml with KBR (=erythrocyte stock solution). A 1/7 dilution of this cell concentration (the final concentration of cells in the assay) gave an $O.D._{413}$ on lysis with 1% saponin of 0.66 +/- 0.06 (95% confidence limits, mean of 10 determinations). Rabbit anti-sheep erythrocyte antiserum (german government-tested, commercially available under the trade name "Amboceptor", Behringwerke) was then added to the erythrocytes to sensitise them to complement. The optimal amount of Amboceptor varied with the age of the erythrocytes, the batch and the age of the complement preparation, and was periodically determined by a two way titration of amboceptor and complement. For the experiments described in this work about 1.25 µl/ml erythrocyte stock solution was required (=sensitised erythrocytes). Sensitised erythrocytes were prepared fresh every week.

Complement dilution

Guinea pig complement was obtained from Behringwerke and stored in the cold. Each batch was only used up to one week before the expiry date set by the manufacturer. To determine the amount to use in the assay system between 0.2 and 0.3 ml of a 1/150, 1/200 or 1/250 dilution in KBR (depending on batch and age of batch) was made up to 1.2 ml with KBR in a small Eppendorf tube (1.7 ml max. volume) and incubated for 18 hr in the cold. 0.2 ml of sensitised erythrocytes were added and the preparation incubated for 1hr at 37°C. The tubes were then

chilled and centrifuged (2 min in an Eppendorf table centrifuge), the supernatant decanted off and the $O.D._{413}$ determined. A titration curve was plotted and the amount of complement giving 50% of maximal lysis (saponin value) was determined. 1.2 times this amount was then used in the assay and gave approximately 90% of maximum lysis.

Antibody-antigen assay (complement fixation)

The procedure was the same as for determining complement dilution, except that the required amounts of antigen or antibody (each usually in 100 µl of whatever buffer they were contained in) were first mixed and made up to 900 µl with KBR buffer. An amount of complement determined as described above was added and the total volume made up to 1.2 ml with KBR buffer. If serum rather than purified antibody was assayed it was first heated to 60°C for 20 minutes to inactivate any endogenous complement it might contain. Controls with no antigen or no antibody were always included to detect non-specific inhibition of complement. Maximum complement dependent lysis was determined in a sample containing neither antigen nor antibody. Maximum possible lysis was determined by addition of 1% v/v of a 0.1 M solution of saponin to a blank containing sensitised erythrocytes and provided a control that the correct activity of complement and the correct amount of erythrocytes had been added to the assay. Complement fixation (CF) was determined according to the following formula:

$$CF = (1-(O.D._{413}\ \text{Ab-ag}/O.D._{413}\ \text{complement alone})) \times 100$$

M.2.5.1.2.2 ^{51}Cr Release

5.10^8 cells (1ml) of amboceptor sensitised cells were

centrifuged (8000 rpm 30 seconds, Eppendorf table centrifuge) and resuspended in 20 µl 0.9% NaCl, containing 500 µCi (18.5 MBq) $Na_2{}^{51}CrO_4$ (1.6 µg Cr, 2.10^{-6}M) and 4 µl 5x concentrated KBR buffer. They were incubated at 37°C for 1hr and then suspended in 1 ml KBR and centrifuged. The cells were washed by centrifugation and resuspension twice more. After the final resuspension they could be stored in the cold for 2-3 days. Freshly prepared ^{51}Cr loaded cells after this treatment contained between 1.5 and 3.5 10^4cpm/10^6 cells.

The cells were somewhat leaky and were washed twice immediatly before use.

Complement fixation took place in a reaction volume of 200 µl in KBR buffer containing antigen+antibody and between 20 and 50 µl guinea pig complement. Conditions were otherwise as for the haemoglobin release assay. Assay for complement was initiated by addition of 40 µl of freshly washed ^{51}Cr labelled erythrocytes 1:10 diluted in KBR (2.10^6 cells). The initial cell content of the assay was determined by counting the whole tube and the cells were then incubated at 37°C for 1 hour. The reaction was stopped by chilling and centrifugation. ^{51}Cr release was determined from 200 µl of the supernatant.

Thus total $cpm_{released} = cpm_{sample} \times 1.2$.
Using freshly prepared cells background lysis was less than 1000 cpm compared to maximum release by complement of approximately $3. 10^4$ cpm.

M.2.5.1.3 Immunoradiometric Assay (IRMA)

The following remarks apply to all solid phase assays using radioactive reagents. All assays were performed in V-form PVC 96 well microtiter plates (Cooke). Antigen was bound for 1hr on ice, usually in a volume of 100 µl. Antigen was removed by aspiration and the plates blocked using 300 µl of 1% BSA for two hours on ice. The plates were then washed with 2 x 300 µl PBS. 110 µl of antibody containing solution was added and the plates incubated overnight, either in the cold

or on ice. Antibody solution was removed by aspiration and the plates washed with 2 x 300 µl PBS. ^{125}I labelled second antibody or ^{125}I labelled protein A, between 10^5 and 2.0 10^5 cpm in PBS containing 0.1% Triton was added in 120 µl and the plates incubated for a further 12 hours. Radioactive antibody was aspirated into an appropriate waste container and the plates were washed two further times with 300 µl PBS containing 0.1% Triton. Great care was taken to ensure that the wells were completely completely emptied during the last three aspiration steps. The plates were then dried and the wells separated from the plate by use of a hot wire. Care was taken to perform this step in a fume cupboard since PVC fumes are known to be toxic and can lead to severe skin irritations if the appropriate precautions are not taken. The separated wells were counted in using the ^{125}I "open channel", which counted with about 85% efficiency. For especially precise measurements the gamma counter tubes in which the detached wells were placed were counted empty, and a correction made for the contamination in these tubes which inevitably accumulated after they had been used more than 10 to 20 times.

M.2.5.1.4 EnzymeLinkedImmunoadSorbentAssay (ELISA)

A variety of antigens and antibodies were assayed by the ELISA method. The enzyme linked to the detector reagent (anti-antibody or avidin) was always phosphatase.

Mouse antibody was detected using phosphatase-labelled $(Fab')_2$ fragment of rabbit anti-mouse antibody directed against both the heavy and light chains of mouse IgG (abbreviation: $(Fab')_2$ RAM (H+L)). Where not otherwise indicated this was used at a dilution of 1/500. Rabbit antibody was detected in two stages. Biotinylated goat anti-rabbit antibody was first bound to the rabbit antibody, washed and then avidin phosphatase was bound to the solid-phase bound biotin. The binding of avidin to biotin should be nearly

stoichiometric, because the K_{eq} of the avidin-biotin binding reaction is extremely high (> 10^{14} M^{-1}, GREEN 1975).

With respect to incubation times for the binding reactions (including binding to the surface of microtitre plate wells) and the concentrations of the immununological components there are no "standard procedures", because many ELISA experiments involved testing the effect of altering these assay variables. Details of some of the more common procedures are given in the following three sections M.2.5.2.1 - M.2.5.2.4). However, the following steps were common to most assays.

Apart from antigen, which was usually diluted in PBS, all other assay components were diluted in Borate Saline (BS) containing 0.1% BSA.

BS had the following composition:

Substance	Amount (g/l)	Concentration (M)
Boric Acid	6.2	0.1
Borax ($Na_2B_4O_7 . 10H_2O$)	9.53	0.05(Na^+) 0.025(tetraborate)
NaCl	4.4	0.075

pH adjusted to 8.2 with concentrated HCl

Unless otherwise indicated incubations were carried out at room temperature using plates with lids lined with moist tissue paper and sealed with parafilm. Antigen was usually applied in a volume of 100 µl and was blocked with 300 µl 0.1% BSA in BS for at least 30 min. After this step the plates were washed twice with BS and after each of the binding reactions the plates were washed twice with 300 µl BS - 0.1%BSA. Antibody containing solutions were removed carefully and thoroughly by aspiration to avoid cross-contamination, but after wash steps the plates were usually emptied by inverting the plates with a quick flick of the wrist. 0.1% Tween 80 was included in the two wash steps following the binding of phosphatase coupled protein to the plates.

After the Tween washes the the plates were washed once in substrate buffer (SB) which was made up as follows:

Substance	Amount/l	Concentration (M)
Diethanolamine	96 ml	1.0
$MgCl_2$	47 mg	$5x\ 10^{-4}$

adjusted to pH 9.8 with concentrated HCl.

The substrate for phosphatase was the disodium salt of p-nitrophenolphosphate made up at a concentration of 1 mg/ml (2.7 mM) in SB. The assay reaction was started by addition of this solution to the microtitre plate wells.

The substrate solution was always made up fresh using substrate which was stored as the solid at -20°C in an air-tight container and gave an almost colourless solution. Other commercially available salts of dinitrophenolphosphate or inappropriately stored batches of the disodium salt gave solutions which were noticeably yellow indicating that they were already hydrolysed to an unacceptably large extent.

The hydrolysis of substrate in each individual well was followed using a Dynatech MR 580 Microelisa reader to measure the difference in absorbance between the wavelengths 490 nm and 405 nm and between 450 nm and 490 nm. All absorbance differences were read relative to the absorbance difference of the substrate solution in a well which had only been exposed to wash buffer, which was automatically set to zero; i.e. only absorbance differences resulting from any treatment to which the well surface had been exposed and not those resulting from intrinsic hydrolysis of substrate were measured. Intrinsic hydrolysis was very low, but was detectable after long periods. Controls for non-specific binding of assay components were always included as part of the assay design.

Usually several (6 or 7) readings were taken at time intervals appropriate to the velocity of the reaction being followed (the time intervals might vary from 5 minutes to 5

hours). The absorbance data, date and the time of day (to the nearest minute) were communicated in electronically coded form to a microcomputer and stored on a magnetic medium (floppy disk cf. M.6.3). At the end of the experiment the data was processed electronically (see M.7.6) to give rate of reaction data for each experimental treatment.

M.2.5.2 Assays for specific substances

M.2.5.2.1 Histone 1

In all experiments in either IRMA or ELISA in which histone 1 was assayed, either in the form of the pure protein or as component of a mixture of proteins, after the initial binding step, which was usually carried out in PBS, all buffers except the ELISA substrate buffer contained 1 M NaCl in addition to any NaCl they already contained. This addition was neccessary to depress high levels of non-specific binding or to enhance the assay response.

M.2.5.2.2 Histone 1 in situ in fixed Chromatin

Fixed or unfixed soluble chromatin, prepared as described in section M.3.3. was diluted to a concentration of 1 $O.D._{260}$/ml. After addition of lysine (1 mM) to block any reactive glutaraldehyde residues and BSA to 1% to block non-specific binding, 14, 7 or 3.5 µg of anti-H1 (402) was added to each of 3 1 ml aliquots. After mixing by inversion and incubation for 30 minutes on ice 6 10^5 cpm ^{125}I protein A was added and incubation continued for 1 hour. Chromatin was precipitated by the addition of 1 M $CaCl_2$ to 6 mM and the pellet spun down by centrifugation for 2 min. in an Eppendorf centrifuge. The supernatant was carefully removed and radio-activity in the pellet was counted. Controls for the binding of Protein A without chromatin or antibody were included.

M.2.5.2.3 Mouse IgG in Hybridoma Supernatants

To calibrate the assay (IRMA or ELISA) a series of "pseudosupernatants", containing known amounts of polyclonal mouse serum IgG between 1.6 and 100 µg/ml was produced from a stock solution of mouse serum IgG whose concentration had been determined spectrophotometrically (M.1.1.2), by dilution in fresh (unused) serum containing cell culture medium, identical to that in which hybridomas were cultured (usually RPMI containing 20% FCS).

Each of the pseudosupernatants and the hybridoma culture supernatants whose IgG content was to be determined was diluted 1 to 1000 in PBS. Medium alone was also diluted in this way to provide a correction for non-specific detector binding. The diluted solutions were used to coat the surface of the microtitre plate wells with mouse IgG, which in this case served as antigen. The length of this incubation was usually 30 minutes but could be varied, provided that both calibration and unknown solutions were incubated for the same period of time.

The coated wells were blocked, washed, incubated directly with detector (^{125}I RAM Fab or with Phosphatase linked $(Fab')_2$RAM(H+L)) and the assay response determined in the usual manner.

A calibration curve was constructed by plotting the assay response of the pseudosupernatants (corrected for "non-specific" binding of detector to medium components) against the concentration of mouse serum IgG which they contained. It was convenient to do this on a log-log scale and gave a straight line. The concentration of mouse serum IgG which gave an assay response corresponding to that of hybridoma supernatants could then be read from this graph. The value of "µg mouse serum IgG equivalents/ml" thus obtained could be used as an approximate (correct to within a factor 2 (IRMA) or 5 (ELISA)) measure of the absolute amount of IgG present in these supernatants (cf. R.8).

M.2.5.2.4 Drosophila antigens in complex mixtures

Drosophila nuclei were prepared from Kc cells prepared according to SAUMWEBER (1980) were kindly provided by H.SAUMWEBER and were sonicated extensively (6 times 20 seconds on ice, with 1 minute cooling pauses, using a Branson sonifier with a macrotip operated at full power) before use. The concentration of nuclear protein was measured using the Bradford assay after sonication of nuclei. The sonicated extracts were diluted in PBS.

RNP enriched extracts and fractions obtained therefrom by centrifugation in sucrose gradients were diluted in PBS by a factor (determined by preliminary experimentation) to bring them into a range in which the amount of antigen limited the assay response.

Where possible purified monoclonal antibodies whose concentration had been determined spectrophotometrically at 280 nm were used to detect antigen. Unless otherwise indicated their concentration was adjusted to 1 µg/ml. Polybuffer containing antibodies (see R.3.3.3) were used for some assays, but controls showed that this contaminant did not affect the assay response. If purified antibody was not available hybridoma supernatants were used whose concentration had been adjusted to 1 "µg mouse serum IgG equivalent"/ml. Antibody dilutions for IRMA assays were made using cell culture medium, and any non-specific assay response caused by binding of serum to Drosophila components was corrected by using the appropriate controls. In ELISA use of serum led to very high non-specific assay response values and for this reason BS - 0.1% BSA was used to dilute antibody.

M.2.5.3 Western Blots

In early experiments (autoradiographic detection) proteins which had been separated by electrophoresis in SDS-

polyacrylamide gels were transferred to nitrocellulose sheets according to RISAU (1980). Rabbit antibody used to detect nitrocellulose components was visualised using ^{125}I labelled protein A.

In later experiments (immunoenzymatic detection) protein was electrophoretically transferred to nitrocellulose (TOWBIN et al., 1979) using an apparatus constructed at the Max Planck Intitute für Virusforschung in Tübingen. After blocking with 1% BSA in PBS all steps were performed in BS - 0.1% BSA. Rabbit antibody was detected immunologically using biotinyl labelled goat anti-rabbit antibody and, after washing, avidin phosphatase was attached to the bound antibody. The immune complexes were visualised by using 1 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt)), dissolved in 0.1 M carbonate buffer pH 10.2, as substrate. Recently, the concentration of BCIP has been reduced to 0.5 mg/ml without any apparent loss of sensitivity. The hydrolysis product of this substrate gives a beautiful deep sky blue precipitate.

M.2.6 Immunocytological procedures

M.2.6.1 Direct and Indirect immunofluoresence staining of Drosophila Kc cells

Drosophila Kc cells (ECHALIER and OHANESSIAN 1970) adapted to grow in serum free D20 medium were grown on sterile glass microscope slides in the presence of 4 µg/ml Cholesterol to promote cell spreading (FALKNER et al., 1981). Cells were washed once in cold PBS and fixed in 3.7% formaldehyde for 1 minute at room temperature. The following extractions were then carried out using solvents cooled to -20°C; 10 min methanol, 10 min acetone/methanol (1:1), 10 min methanol. These steps served to make the cytoplasm and nuclei of the fixed cell permeable to antibody OSBORN et al., (1977). After rinsing in PBS the cells were mounted in PBS containing 58%

glyerol under coverslips. The slides could be stored frozen for several months without loss of any of the nuclear antigens tested although their morphology did begin to deteriorate after after three months. For indirect immunofluoresence staining frozen cells were thawed and rinsed in PBS for 30 minutes, incubated under a cover slip in 5 µl of a 10 µg/ml solution of monoclonal antibody diluted in RPMI containing 20% FCS for 30 min. The cover slip was removed and the microscope slide rinsed using running tap water. The cells were incubated twice in a large excess of PBS for 15 minutes to remove any loosely bound antibody. Finally, the cells were incubated for 30 min in the dark under a coverslip in 5 µl of a commercially obtained FITC-labelled rabbit antibody preparation directed against both the heavy and light chains of mouse (IgG RAM (H+L)) diluted 1/40 or 1/50 in PBS - 0.1% BSA. The coverslip was removed and the cells were washed twice as above and then mounted in PBS containing 58% glycerol.

For direct immunofluoresence staining the cells were incubated under the same conditions as for the first antibody binding reaction in indirect fluoresence, except that the concentration of directly labelled antibody was 30 µg/ml.

M.2.6.2 Direct and Indirect immunofluoresence staining of Drosophila Salivary gland polytene chromosomes

Polytene chromosome squashes and indirect immunofluoresence staining was performed as described in SAUMWEBER et al., (1980), except that Buffer A* (HEWISH and BURGOYNE 1970, RISAU et al., 1983) rather than PBS was used for all incubations.

Direct immunofluoresence staining was usually performed using a mixture of fluorescently labelled monoclonal antibodies one fluorescing red, the other green. The concentration of each of the antibodies was 30 µg/ml, otherwise the conditions were the same as for the initial antibody binding reaction in indirect immunofluoresence

staining. p-Phenylenediamine at a rate of 1 mg/ml was added to the mounting medium to reduce bleaching of FITC by illumination (JOHNSON and DE NOGUEIRA ARAUGO 1981).

M.2.7 Sources of Immunological Reagents

The quality of immunological preparations varies considerably from supplier to the supplier.

The suppliers for the reagents used in this work were as follows:

ELISA and Western Blot Reagents:
all these reagents had been affinity purified

Phosphatase labelled $(Fab')_2$ goat anti-mouse (H+L)
Biotinyl labelled $(Fab')_2$ goat anti rabbit-antibody
Phosphatase labelled avidin
Rabbit anti-mouse IgM (μ chain specific)

from MEDAC, Fehlandstr. 2, 2000 Hamburg 36
importers for the firm of TAGO, Burlinghame, CA. USA
and E-Y Laboratories, San Matheo, CA. USA (biotin-Avidin reagents)

Phosphatase labelled Goat anti-mouse $(Fab')_2$
Phosphatase labelled Goat anti-mouse (H+L)

from Jackson Immunoresearch, Avondale, PENN. USA (imported direct).

Immunofluoresence reagents
(not affinity purified)

FITC-labelled rabbit anti-mouse H+L
from MILES, Kennedyallee 32, 6000 FRANKFURT

Ouchterlony

Antisera against mouse IgG subclasses
NORDIC

M.3 Preparation and Fractionation of Nuclei and Nuclear Extracts

M.3.1 ^{35}S Labelled Nuclei from Drosophila Kc Cells

Cells from a 1 l stirred culture growing in D-20 medium (ECHALIER and OHANESSIAN 1970) were harvested by centrifugation in sterile centrifuge bottles and resuspended in D-20 medium lacking yeast extract but containing 1 mCi ^{35}S labelled methionine (AMERSHAM) and incubated overnight with stirring. Nuclei were prepared according to RISAU (1980).

M.3.2. hnRNP and hnRNP protein enriched extracts

hnRNP enriched extracts and and fractions therof obtained by centrifugation in sucrose gradients were kindly donated by W.RISAU who prepared them according to the procedures described in RISAU et al., (1983).

Another form of hnRNP protein enriched fraction was kindly provided by M.FRASCH and consisted of pooled fractions from the top of a sucrose gradient to which Kc cell nuclei digested in RNAase and extracted in 0.5 M NaCl had been applied (M.FRASCH pers. comm.).

M.3.3 Preparation and Fixation of Calf Lymphocyte Chromatin

Nuclei from calf lymph node lymphocytes prepared according to RENZ et al., (1977) were kindly provided by M.RENZ. They were suspended im 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM TEA pH 7.5 at a concentration of 300 $O.D._{260}$ units/ml and digested with 50 units micrococcal nuclease/200.$D._{260}$ units for 30 min. on ice. The digestion was stopped by the addition of an equal volume of 140 mM NaCl, 10 mM EDTA and stood for 5 minutes. Soluble Chromatin was obtained in the supernatant after centrifugation at 3000 rpm

for 5 minutes in the HB4 rotor of a SORVALL centrifuge. Micrococcal nuclease was removed by passage over a Biogel A 1.5 column equilibrated in 70 mM NaCl, 1 mM EDTA, 5 mM TEA pH 7.5. Pooled material from this column was fixed with various concentrations of glutaraldehyde at a chromatin concentration of 6 $O.D._{260}$/ml for 1 hour on ice. The glutaraldehyde was from a newly opened bottle of a 25% stock solution (MERCK - type 12179) and was diluted immediately before use. Fixation was ended by passage over a Sephadex G50 column equilibrated in the same buffer as the Biogel column.

M.4 Nucleic acid Preparation

M.4.1 DNA Supercoils

Supercoiled ColE1 plasmid DNA was prepared from the E. coli strain P1628 (ColE1, thy^-, lac^-). This strain was a kind gift of A. KLEIN. The cells were grown in L-Broth supplemented with 0.1% (w/v) glucose and 2 µg/ml thymine. Cells were aerated and kept in suspension by bubbling air through the medium. When the cells reached a concentration of 2 10^8 cells/ml ($O.D._{420}$ 1.6 - 2.0) chloramphenicol was added to a final concentration of 200 µg/ml. At this concentration of chloramphenicol the DNA replication of the bacterial genome but not of plasmid DNA is inhibited (CLEWELL 1972) so that plasmid DNA accumulates (1200 copies/cell, approx. 45% of the total DNA in the cell). Radioactive DNA was obtained by adding either (2-^{14}C) thymine at a concentration of 0.9 µCi/ml (33.3 MBq) or (6-^{3}H) thymine at a concentration of 1.5 µCi/ml (55.5 MBq/ml) 1 hour after chloramphenicol addition (when bacterial genome DNA synthesis had ceased completely).

DNA was prepared as follows (amounts for 400 ml of cells): cells were harvested by centrifugation and resuspended in 6 ml ice cold 10 mM Tris pH 8.0, 25% (w/v) sucrose. Lysozyme (0.9 ml of a 10 mg/ml solution in 250 mM Tris pH 8.0) was added and the suspension agitated for 45 seconds in a 42°

water bath then placed on ice for 5 minutes. 3.2 ml 0.25 M Na-EDTA pH8.0 was added and the cells left for a further 5 minutes on ice. Addition of 5 ml 4% (w/v) Triton X-100, 50 mM Tris-Cl pH 8.0, 62.5 mM EDTA, led to complete cell lysis. The very viscous lysate was mixed gently with the tip of a glass pipette and after a further 20 min on ice was centrifuged for 15 min at 17K rpm in a SORVALL centrifuge using an SS34 rotor. The supernatant (cleared lysate) containing the plasmid DNA was decanted and the pellet discarded.

All subsequent steps were carried out using sterilised solutions to avoid contamination by nucleases. Supercoiled DNA was separated from other cellular nucleic acids in ethidium bromide (EtBr) containing CsCl density gradients by virtue of its different buoyant density. 9.74 ml cleared lysate, 9 g CsCl and 0.36 ml 10mg/ml EtBr were mixed and centrifuged in a 50Ti rotor for 42 hours at 36K rpm at 15°C. A sealed connection to a light oil resevoir was made at the upper end of the centrifuge tubes, their bottoms were pierced and fractions collected by pumping oil onto the tops of the tubes in a controlled way. Fractions containing supercoiled DNA were pooled and extracted 3 times with isopropanol equilibrated with a saturated solution of CsCl in TEN (10 mM Tris pH 7.5, 1 mM EDTA, 50 mM NaCl). Pronase from a stock solution kept at room temperature was added to 50 µg/ml and the material dialysed against TE (10 mM Tris pH 7.5, 1 mM EDTA) for 2 x 3hr in the cold. A further 50 µg/ml of Pronase was added and the mixture incubated at 37° for 30 min. The preparation was extracted twice with phenol (equilibrated with TE), once with phenol-chloroform and dialysed exhaustively against TE.

This preparation contained large amounts of tRNA. After concentrating the preparation by precipitation with 3 volumes of Ethanol (-20°C, overnight after adjusting the salt concentration to 200 mM with NaCl), the tRNA was removed by chromatography over Sepharose 4B equilibrated in TEN.

Electrophoretic analysis of the final preparation either in 0.8% vertical agarose gels (GREENE et al., 1974) or 0.5%

horizontal ones (KELLER 1975) showed the final preparation to comprise both supercoiled and relaxed circular molecules.

M.4.2 Supercoil-Histone 1 complexes

Complexes were formed by mixing H1 and DNA at the appropriate salt concentration in a solutio buffered with 5 mM Tris, 5 mM EDTA pH 7.5. The sedimention properties of the complexes were analysed by their sedimentation velocity in 5-20% sucrose gradients in the same buffer (at various salt concentrations). The gradients also contained 1 mg/ml BSA, which was important to prevent the adsorbtion of radioactive DNA and especially radiolabelled protein to the walls of the centrifuge tubes (polyallomer type). Centrifugation was at 4°C in an SW40 swing-out rotor at 39,000 rpm for 6 hours. In some experiments equivalent centifugation conditions were used, calculated according to the formula:

$$\omega_1^2 \, t_1 = \omega_2^2 \, t_2$$

where ω = angular velocity of rotor in rpm
and t = time of centrifugation

Fractions were collected from the top of the tube and their $O.D._{260}$ obtained by using an ISCO fraction attached to a flow through spectrophotometer.

M.5 Radioisotopic Techniques

M.5.1 Radiolabelling of Proteins

H1 was substituted at lysine residues with ^{125}I Iodotyrosine at a substitution rate of about 0.5 mole ^{125}I/mole by using Bolton-Hunter reagent (AMERSHAM), N-succinimidyl-3-(4-hydroxy 5-(^{125}I)-iodophenyl propionate). Labelling was carried out according to the manufacturers instructions.

Protein A was substituted with ^{125}I at ?histidine?

residues (cf. R.7.3.2.1) using chloramine T according to the following procedure.

The protein A obtained from PHARMACIA was not homogeneous. A fraction from the main peak obtained by chromatography on a Biogel A 1.5 column was used for the experiments reported in this work. All reagents except ^{125}I were dissolved in 50 mM phosphate buffer pH 7.0. All reagents were made fresh in buffer precooled to $0^{0}C$. The reaction was carried out on ice. 8 µg of the fractionated protein A in 13 µl was added 0.5 mCi ^{125}I (carrier free, Na salt in 1 mM NaOH), and 6 µl 1 mg/ml Chloramine T. After briefly mixing with a pipette tip the iodination mixture was incubated for 30 seconds and the reaction was then stopped by the addition of 6 µl 3mg/ml $Na_2S_2O_5$ and 6 µl 10 mg/ml KI was added to reduce adsorbtion of unreacted ^{125}I Iodide. Iodinated protein was separated from unreacted ^{125}I by chromatography over a 7 ml G50 (fine) Sephadex column equilibrated in 10mM Tris, 10 mM NaCl, 0.5% (w/v) Triton and 0.2 mM PMSF. 16 drop fractions were collected, 4 µl aliquots counted, the peak fractions pooled, diluted to a concentration of approx. 10^{5} cpm/µl in elution buffer, divided into several (20-40) aliquots and stored frozen at $-20^{0}C$. For some later preparations the column elution buffer was 0.1% BSA in PBS rather than the triton containing buffer described here. Usually more than 80% of the input ^{125}I was recovered in the protein containing fractions and >98% of the counts in these fractions were acid precipitable.

M.5.2 Counting and Double Label Experiments

^{125}I and ^{51}Cr were counted in an Intertechnique gamma counter with efficiencies of 85% and <10% respectively (manufacturer's figures). ^{3}H, ^{14}C and ^{35}S were counted in a three channel Packard Liquid Scintillation Spectrometer. PPO dissolved in toluene (5g/l) was used as scintillator to count

dry samples. Aqueous samples were counted using PPO (0.7g/l) dissolved in a 30% Triton- 70%Toluene mixture, using 9 parts of this mixture and 1 part aqueous sample to form an emulsion. When counting sucrose gradient fractions directly aliquots were diluted to reduce the sucrose concentration below 10%. This avoided precipitation of sucrose and quenching. The total volume counted was usually 2ml.

In $I^{125}/^{14}C$ double label experiments the whole ^{125}I containing sample was usually first counted non-destructively in the gamma counter. After suitable dilution ^{14}C was counted by liquid scintillation using amplifier and window settings such that less than 1% of the ^{125}I dpm spilled over into the ^{14}C channel. The counting efficiency of ^{14}C under double label conditions was only 25%. It would also be possible to count ^{125}I in the liquid scintillation counter using the Auger electron of the iodine isotope. However at maximum amplification and window settings at most 65% of the counting efficiency of the gamma counter could be acheived. ^{14}C was counted with nearly 100% efficiency under these conditions, causing innaccurate estimation of ^{125}I at high $^{14}C/^{125}I$ ratios.

M.6 Microscopy and Instrumentation

M.6.1 Fluoresence Microscopy

For routine purposes a ZEISS Universal microscope using a 40x Neofluar phase contrast objective, and fluoresence illumination was from a HBO 100W/2 lamp. Epifluoresence from FITC stained preparations was observed at wavelengths above 520 nm by using a LP 520 nm cut-off filter for the emitted light (fluoresence), a 435-490 nm band pass filter and an FT510 filter for the exciting light. Epifluoresence from TRITC stained preparations was observed at wavelengths >590 nm using the cutoff filters recommended by Zeiss and a 545 nm glass filter to obtain the exciting light.

For quantitative image analysis a ZEISS Axiomat with an inverted objective stage was used. Preparations were examined with the 100x Phako oil immersion objective (N.A. 1.30). An HBO 100W/2 lamp was used for epifluoresence illumination. Filter pairs (incident wavelength(s):emitted wavelength(s)) were 435-490nm:>520nm and 547nm:>590nm for FITC and TRITC respectively.

Phase contrast micrographs were photographed on Ilford HP5 film. Colour slides were photographed on Kodak Ektachrome film

The production of video images is described in the next section.

M.6.2 Quantitative Image Analysis

Video images were obtained with a HAMAMATSU image intensifying NOCTICON camera and digitised using HAMAMATSU equipment. The resultant image was stored in an electronic frame store and could be recalled or manipulated with the aid of a PDP11/03 computer. The image was digitised at a resolution of 512 x 512 pixels and 256 intensity levels. To reduce background noise the mean of 10 images was taken.

To produce good video images, the intensity of the exciting light had to be adjusted to bring the intensity of the fluoresence into the range below which the camera signal saturated. Gross adjustments were made by using neutral density and blue glass filters. Fine adjustments were made as follows.

The absolute level of fluoresence appeared to vary somewhat between experiments performed on different days. The following procedure was used to bring stained regions into the measureable range of the digitiser. i) An arbitrary amplification factor for the image intensifier was chosen which was kept constant from experiment to experiment. ii) The first preparation to be examined at the beginning of a

particular experiment was quickly scanned and a very strongly fluorescently stained region found. The **illumination** was then adjusted, both for red and green fluoresence, by means of an adjustable diaphragm, to give a signal strength of 180 - 200 machine units (approx. 80% of the maximum) from the digitiser. These levels of illumination were used for all subsequent experiments on that day. Obviously, since the absolute value of the measurement was dependent upon the properties of the locus chosen to set the illumination level, the numerical values of fluoresence intensity could only be exactly compared between data gathered from a single experiment.

To measure K_c cell staining intensities at different concentrations of antibody, a FORTRAN program written by the late R.BURBERG was used. The program computed the mean intensity per pixel for areas of cells with fluoresence intensity values greater than some arbitrary value set by the operator. Although there may have been small differences in the optical path from preparation to preparation, controls showed that the fluoresence intensity from identically stained preparations on different slides was reproducible if identical conditions of illumination were used (diaphragm openings, coloured filters).

The intensity of the fluorescence staining along the length of the polytene chromosomes was obtained from the digitised images by constructing Maximum Intensity Profiles (MIPs) of red and green fluoresence respectively. Using another FORTRAN program written by the late R.BURBERG the operator places 9 pixel high bars on the image at positions determined by the contour of the chromosome. The program then connects the bars (only a line connecting their centres is shown on the monitor on which the stored image is displayed) and locates the pixel with the maximum intensity within the 9 pixel width of the polygon thus formed for each pixel of its length. The intensities thus measured are then displayed on the monitor in the form of a graph with intensity represented by the height of the y axis and the x axis showing the

position on the polygon. The intensity data was also output to a printer for later numerical analysis if required.

Images from the monitor (Visual Display Unit) were photographed on Agfa Pan F-15 film using an exposure time of approximately 1 second. Because of a quirk of camera design, the monitor pictures are horizontally distorted by a factor of about 1.25. This effect could be reversed photographically by tilting the paper upon which images were printed by an appropriate angle relative to the negative.

The correlation between MIPs was examined by the use of scatter plots in which the intensity at each point in the MIP constructed from the image of the red fluoresence was plotted against the corresponding point from the image of green fluoresence. The red and green images were shifted with respect to one another by between 1 to 3 pixels depending upon the experiment. Controls in which the polarity of the image of a fluorescent chromosome was reversed (by turning the microscope slide through 180^{o} with respect to its long axis) showed that this shift was a property of the optical system and not of the chromosomes. i.e. the red image was always shifted left with respect to the green image. The red and green data sets were brought into register with one another, ordered with respect to the intensity of one of the images and the scatter plot printed with the aid of a MICROSOFT BASIC program (available on request).

M.6.3 Electronic Data Storage and manipulation

Data processing was carried out either on a TRIUMPH-ADLER Alfatronic P1 microcomputer with 48 KBytes random access memory (RAM) or on a Heath/Zenith H89 with 64K RAM. The Alphatronic was equipped with a single 5 1/4 disk drive which could store up to 160K characters of data and the Heath/Zenith machine with 3 drives recording a maximum of up to 700K characters of data. Both machines were operated under the CP/M

operating system and were connected to a printer and monitor.

Data from the MICROELISA reader could be transferred to the Alfatronic using a simple two wire connection (data line and ground). Data was automatically sent from the ELISA reader at 9600 Baud (bits per second) whenever a reading was made. A machine language (compiled) program supplied by the firm of GLASER set up the input/output port of the microcomputer to receive data at 9600 Baud (bits per second), detected when data was received, and stored received data in the computer's memory until an operator determined amount of data (maximum 1270 ASCII characters, the maximum which the ELISA reader could send) had been counted. The received data was then displayed on the monitor of the microcomputer (optionally it could be printed out), to confirm that it had been received correctly, and was then recorded in magnetic form on the surface of a floppy disk under a file name of the operators choosing.

M.6.4 Spectrophotometry

Where not otherwise indicated O.D. values were measured using a ZEISS PM-Q single beam spectrophotometer and 1 cm pathlength cuvettes.

Spectra were measured using a GILFORD single beam spectrophotometer which automatically altered the wavelength of the light beam. A special two chambered cuvette was used for spectral measurements, one chamber containing a reference solution and the other the solution whose spectrum was to be measured. A special attachment transported this cuvette rapidly to and fro in front of the light beam as the wavelength changed and adjusted the slit width of the instrument to give a zero O.D. from the reference half of the cuvette. An analog electrical signal proportional to O.D. was sent to one channel of a two channel chart recorder. The other channel received a small low voltage spike for every nm of

spectrum scanned.

The time course of absorbance changes in up to 4 cuvettes could also be recorded in the GILFORD using an automatic sample changer attachment.

O.D. values from chromatography columns were measured using a GILSON flow through spectrophotometer with the smallest volume flow through cuvette. The values obtained from this instrument were inaccurate because it proved difficult to calibrate (for reasons which are not understood the absorbtion values appeared to change with flow rate) and because, at least in the cold, the response of the instrument saturated below 2.O.D. resulting in erroneous readings at high O.D. values.

M.7 Statistical and Computational Methods

M.7.1 Particle Counting

A Neubauer Haemocytometer with grid square sizes of 0.0025 mm^2 and 0.0625 mm^2 arranged in clusters of 16 was used. The cover slip clearance was 0.1 mm and a heavy rigid "haemocytometer" cover slip was used. When possible particles were diluted to give approximately 100 particles/16 grid squares (either large or small, depending on particle size and/or concentration). One cluster of 16 grid squares was the usual unit area which was counted. Cells lying across the upper and lefthand borders of the grid cluster were included and those lying across the bottom and righthand borders were excluded from the count.

To estimate any population at least 8 separate "16 clusters" containing at least two separate aliquots of the particle suspension were counted. The mean of this sample was used to estimate the population mean.

The conversion factors to convert counts to particles/ml may be calculated as follows:

Volume above cluster = N.A.H

where N = number in cluster, A = grid square area,
H = coverslip clearance

for small grid = 0.004 mm^3

for large grid = 0.1 mm^3

1 ml = 10^3 mm^3 therefore factor for

small grid cluster = $2.5\ 10^5$

large grid cluster = 10^4

M.7.2 Estimation of means, standard errors and confidence limits

The term "mean" in this work is used to refer to the arithmetic mean of a sample of data with n elements x_i (i=1....n) and is represented by the notation $\bar{x}$ (spoken: x bar) where $\bar{x}$ is defined as:

$$\bar{x} = \left[\sum_{i=1}^{n} x_i\right]\left[1/n\right]$$

The sum of squares SS of the deviations from the mean is defined and was computed as:

$$SS = \sum_{i=1}^{n} x_i^2 - \frac{\left[\sum_{i=1}^{n} x_i\right]^2}{n}$$

The variance of a sample s^2 is defined as:

$$s^2 = SS\ (n/n-1)$$

The standard deviation (S.D.) is given by:

$$S.D. = \sqrt{s^2}$$

The S.D. gives a measure of the variability of the data, and provided that the sample size is given allows other

statistics to be calculated. However, the (statistical) reliability of data is not immediately obvious from this parameter precisely because of the dependence of sampling statistics on sample size.

An intuitively more understandable measure of statistical reliability is the confidence interval. The P% confidence interval describes the range of values within which, with a probability greater than P%, the mean of the sampled **population** is likely to lie.

The concept of a "population" is somewhat abstract in this context. It can perhaps be made clear by the following, reductio ad absurdum, example. A fraction is obtained in the course of an antibody purification, and its concentration is to be estimated. If a destructive assay is used for the estimation (e.g. ELISA) it would, in principle, be possible to repeat the assay so many times that the whole fraction would be used up. This number of repetitions would be the population size. If the assay were non-destructive (e.g spectrophotometric) the population of estimates would be infinite. If a very large number of assays can be made the estimate may be made very precise, the 99.9% confidence limits might perhaps be a fraction of 1% of the mean estimate. However, this estimate is of little use if there is no more material to be used for experimental purposes.

The idea of sampling and of confidence limits is to be able to estimate the true mean of the population using only a small proportion of the material available for sampling. The estimate will be imprecise because there will be variation between individual measurements, but the statistics I suggest employing allow an accurate estimate of that imprecision. For example it allows an unambiguous answer to a question such as "is the chance that my estimate is in error by a factor 3 more than 1 in 20?".

The distribution of means of samples of size n is known to follow STUDENT's t distribution for n-1 degrees of freedom (d.f) (where n is the number of measurements in the sample)

for a number of different distributions (PARL 1967; POLLARD 1977). The confidence interval is computed by using $\bar{x}$, the sample mean as the **maximum likelihood estimator** of the population mean, and the sample variance s^2 as the estimator of the population variance. The confidence limits C.L. of the mean estimate at probability P' is then given by (POLLARD 1977):

$$C.L.(P') = \bar{x} +/- t_{(100-P')/2,n-1} \sqrt{s^2}/\sqrt{n}$$

where $t_{(100-P')/2,n-1}$ is the value on the abscissa of the normalised t distribution ($\bar{x}$=0) such the percentage of the total area under the t distribution for n-1 degrees of freedom at values above this value equals (100-P')/2. I have given the value of t which is to be used this notation because published tables of the value of t use two different conventions. Both are tabulated according to d.f. and P but P may equal either 100-P' or (100-P')/2 (FISHER and YATES, 1957; POLLARD 1977).

The term $S.D./\sqrt{n}$ is often referred to as the standard error (S.E.).

The statistical significance of the difference between two means was also tested with the use of STUDENT's t distribution. The hypothesis was proposed that the difference between the means of populations μ_2 and μ_1 estimated by two different samples with means $\bar{x}_1$ and $\bar{x}_2$ was zero. Then, given measured variances s_1 and s_2 for each of the two samples and pooling the variance data to provide an estimate of the population variance (a legitimate statistical technique), the t statistic for the difference of the means between the two populations is given by (POLLARD 1977):

$$t = \frac{\{(\bar{x}_2 - \bar{x}_1) - (\mu_2 - \mu_1)\}(n_1 + n_2 - 2)^{1/2}}{\{(1/n_1) + (1/n_2)\}^{1/2} \{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2\}^{1/2}}$$

By definition $\mu_2 = \mu_1$. If the t statistic is greater than $t_{p,}(n_1 + n_2 - 2)$ - note P not P'- then the probability that

the sampled difference belongs to the hypothesised t distribution is **less** than P. If P is very small it is reasonable to reject the hypothesis. i.e. $\mu_2 \neq \mu_1$ and the difference between the means of the two samples probably represents a real difference rather rather than one which might have arisen as a result of variation in the data itself. The confidence limits of the difference between two population means can be calculated by simple rearrangment of the above equation:

$$C.L.(100 - 2P) = (\bar{x}_2 - \bar{x}_1) +/- {}^{t}(n_1 + n_2 - 2),P \quad (Z)$$

where

$$Z = \frac{\{(1/n_1) + (1/n_2)\}^{1/2} \quad \{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2\}^{1/2}}{(n_1 + n_2 - 2)^{1/2}}$$

It should be noted that where data is being sampled over a wide range of values that the variance may be approximately proportional to the mean (i.e a measurement of 1 unit in an assay may have a S.D. of 0.1 associated with it and a value of 100 might have an S.D. of 15). In order to make valid statistical comparisons of such data, especially where data is pooled (e.g. regression, see next section), the data should be transformed to eliminate the numerical proportionality. For instance in the above case a logarithmic transformation is required. The same constraint applies to normalised data (e.g. percentages for which a $\sin^{-1}$ transformation is appropriate) (see POLLARD 1977, chap 14).

There are also some statistics for which no analytical solution has been found, such as the distribution of the quotient between the means of two populations. This distribution can only be found by simulation i.e. the distribution of each of the components of the quotient would have to reconstructed from its mean and variance, the values of dividend and divisor picked pairwise at random from each

of the populations and the form of the resulting population examined.

For small samples the mean and variance were calculated on a pocket calculator on which these operations were standard functions (Texas Programmable 57). For larger samples, or large numbers of different samples, it was more efficient to write a small program for a microcomputer.

One final word of warning. It lies in the nature of sampling that the estimates of some parameters will be incorrect. i.e. for every 20 mean values which I present in the Results part of this work, in 19 cases the true value of the parameter they are estimating will probably lie within the 95% confidence limits, but the the true value of one will probably lie outside these limits.

M.7.3 Line fitting

When a linear relationship was expected between two paired sets of n data points x_i, y_i with means $\bar{x}$ and $\bar{y}$ the regression of y on x was fitted by the method of least squares, by solving the simultaneous equations:

$$W = \frac{\sum_{i=1}^{n} x_i y_i - n\bar{x}\bar{y}}{\sum_{i=1}^{n} x_i^2 - n\bar{x}^2}$$

and

$$V = \bar{y} - W\bar{x}$$

for W, the slope of the regression, and V, the value of y at x=0

The error mean square $\bar{s}^2$ of the variation in y not attributable to the regression is given by:

$$\bar{s}^2 = \left[\sum_{i=1}^{n} y_i^2 - n\bar{y}^2\right] - W^2\left[\sum_{i=1}^{n} x_i^2 - n\bar{x}^2\right]$$

The confidence limits for the slope of the regression W are then given by (POLLARD 1977):

$$C.L.(P') = W \quad +/- \; t_{(100-P'/2),n-2} \; (Z')$$

where

$$Z' = \frac{\bar{s}^2}{\sum_{i=1}^{n} x_i^2 - n\bar{x}^2}$$

and the confidence limits of y at at any value of x are given by (POLLARD 1977):

$$C.L.(P') = \tilde{y} +/- \; t_{(100-P'/2),n-2} \quad \{\bar{s}^2(\mathbf{X}_o.\mathbf{S}^{-1}.\mathbf{X}_o')\}$$

where $\tilde{y}$ = the estimate of y calculated from the regression for x_i and $\mathbf{X}_o, \mathbf{X}_o', \mathbf{S}^{-1}$ are matrices defined as follows:

$$\mathbf{X}_o = \mathbf{X}_o' = (1, x_i)$$

$$\mathbf{S}^{-1} = \begin{vmatrix} n & \sum_{i=1}^{n} x_i \\ \sum_{i=1}^{n} x_i & \sum_{i=1}^{n} x_i^2 \end{vmatrix}^{-1}$$

and $t_{(100-P'/2),n-2}$ is the value of t for n-2 degrees of freedom and probability (100 - P'/2) where P' is the level of confidence for which limits are desired.

The inverse of the matrix was calculated as shown in BATSCHELET (1979, p.518).

Note that if the range of estimates of values for y is estimated using the confidence limits of the slope of the regression alone, this range of y values will be smaller than the if the last formula given above is used, which reflects the variance in both W and V. If the latter confidence limits are plotted graphically they follow a curved path (see e.g. figure in R.7.3.4.2). I draw attention to this point because

only the confidence limits of the slope of the regression are given as a measure of the error of the ELISA rate values.

A computer program written in MICROSOFT BASIC which computes all the regression parameters described here is available on request.

M.7.4 Calculation of Binding Equilibria

The concentration of bound monovalent antigen x_t at time t can be calculated according to the equation (FAZEKAS deSt.GROTH, 1979):

$$x_t = \frac{x_1(x_0-x_2) - x_2(x_0-x_1)\exp\{(x_1-x_2)k_1(t-t_0)\}}{(x_0-x_2) - (x_0-x_1)\exp\{(x_1-x_2)k_1(t-t_0)\}}$$

where x_0 is the concentration of antigen-antibody complexes at zero time (t_0) and x_1, x_0 = the roots of the equation:

$$x_1, x_0 = 1/2\ \{Ab + Ag + K +/- [(Ab + Ag + K)^2 - 4AgAb]^{1/2}\}$$

where Ab = initial concentration of antibody combining sites (2x the molar concentration for IgG)
Ag = initial concentration of antigen
K = Equilibrium concentration defined as k_2/k_1 where k_1 and k_2 are the forward and reverse rates of the reaction respectively. (N.B. this is the inverse of the definition used in section R.3)

The equilibrium concentration is given by the smaller root.

The distribution of antibodies to which 0,1,2 antigen molecules are bound is assumed to a binomial distribution. i.e. given the fraction of free combining sites F =(Ab - x)/Ab and the fraction of bound combining sites B = x_t/Ab, the proportion of antibody molecules with no antigen will be F^2, with one antigen molecule 2FB, and with two antigens B^2, and the total proportion of antibody to which antigen is bound is $B^2 + 2FB$.

A BASIC program (available on request) was written which computed the time course and final equilibrium for any combination of antigen, antibody concentrations, for any equilibrium constant and any combination of forward and backward rates. This was used to obtain the data presented in section R.7.2.1.3.

M.7.5 Calculation of Diffusion Rates

Given a **cylinder** with impermeable ends at whose boundary the concentration of diffusing substance is always zero (i.e. the amount of back diffusion is zero), the fraction F of a substance with an initial uniform unit concentration which will have diffused out of the cylinder at time t, assuming that diffusion can continue until the concentration of the substance reaches zero, is given by the formula (JOST 1957):

$$F = \sum_{v=1}^{\infty} \frac{4}{\xi_v^2} \exp\left[\frac{-\xi_v^2 Dt}{r^2}\right]$$

where ξ_v are the roots of the equation $J_0(x)=0$ where $J_0(x)$ is the zero order BESSEL-function
D=diffusion constant of the diffusing species in units of $cm^2\ s^{-1}$
t=time in seconds for which diffusion has taken place
r=radius of the cylinder in cm

This equation was solved using a program written in BASIC (available on request) which computed the terms of the series until the difference between the series sum for successive values of v was less than 0.0001. Up to the first 60 terms of the series could be computed using the roots of the BESSEL function given in JAHNKE et al., (1960). This resulted in a maximum error of 0.7% for t=0 and for larger values of t where the function converged more rapidly, the error would be negligible.

Given a **sphere** at whose boundary the concentration of diffusing substance is always zero (i.e. the amount of back diffusion is zero), the fraction F of a substance with an initial uniform unit concentration which will have diffused out of the sphere at time t, assuming that diffusion can continue until the concentration of the substance reaches zero, is given by the formula (JOST 1957):

$$F = -\frac{6}{\pi^2}\sum_{v=1}^{\infty} \frac{1}{v^2} \exp\left[\frac{v^2\pi^2 Dt}{r^2}\right]$$

where the variable v is simply the series of integers but all other variables are the same as for the case of the cylinder.

This equation was also solved using a program written in BASIC (available on request) which computed the terms of the series until the difference between the series sum for successive values of v was less than 0.0001. Up to the first 100 terms of the series could be computed. This resulted in a maximum error of 0.3% for t=0 and for larger values of t where the function converged more rapidly, the error would be negligible. An approximation to diffusion out of a sphere only a fraction of whose surface was available for diffusion was made by correcting the power term of the exponent by the corresponding fraction.

The following $D_{20,w}$ values were taken from the literature:

Protein	$D_{20,w} \times 10^7$ cm^2 s^{-1}
IgG	4.3$^+$ (SMITH, 1972)
Protein A	4.3 (BJÖRK et al., 1972)
Avidin	5.98 (GREEN, 1975)
Histone 1	5.5 *

$^+$ This is a mid-range value the reported values for various species

lie between 4.0 and 4.7 10^{-7} cm^2 s^{-1}

* This value was calculated from the molecular weight (23,560) and the sedimentation coefficient ($S_{20,w}$) of H1 (1.237S) reported by HARTMAN et al., (1977) using the formula:

$$D = \frac{SRT}{M(1-v\rho)}$$

where S = Sedimentation coefficient in Svedberg x 10^{-13}
R = Gas constant 8.314 10^7 erg mol^{-1} $^oK^{-1}$
T = Temperature in ^{o} Kelvin
M = Molecular weight
v = partial specific gravity of the protein (= 0.769 for H1, HARTMAN et al., 1977)
ρ = density of solvent (0.998 for water at 20°C)

DAVISON et al., (1954) and HAYDON and PEACOCKE (1968) have reported $S_{20,w}$ values for H1 lying between 1.1 and 1.2.

The diffusion constant of proteins at the temperature and in the solvent in which the diffusion experiment was performed ($D_{t,exp}$) was calculated according to the formula

$$D_{t,exp} = \frac{T_{exp}}{293} \quad \frac{\eta_{exp}}{\eta_{w,20}}$$

The value of η_{exp} was approximated by taking the value of water at the appropriate temperature (HARDY and COTTINGTON 1949). The values of the viscosity and density of salt solutions with concentrations of 140 mM (PBS) differ by only 0.6% and 1.5% respectively from those of water at 20°C (WOLF et al., 1979).

M.7.6 Processing of ELISA data

Data from the MICROELISA reader was stored in the form of ASCII characters as a data file on magnetic medium. The data

from each reading was stored as a separate file and each file was give a name by which it could be identified (either electronically or by the operator) in which was encoded the name of the experiment (a single letter), the wavelength of the reading and the number of the reading in the time course series. The number of characters stored in each file was constant and the characters corresponding to the O.D. values occupied fixed positions in the file.

A program was written in MICROSOFT BASIC which, when run, asked the operator to supply the name of the experiment and the absolute time of day at which the experiment was begun. The file containing the data from the first reading was then automatically located and the data from this file read into microcomputer memory. The parts of the data corresponding to the time of day at which the reading took place and the O.D. values were found by their position. The appropriate characters were converted from the "string variable" data type in which they had to be read from the file (because they were mixed with alphabetic characters) into single precision numeric variables. The time from the beginning of the experiment to the reading was computed and each of the wells in the plate was represented as an element in a 1 x 96 matrix. If a non-numeric value was found where O.D. was expected, which occurred if the absorbance at $O.D._{405} - O.D._{490}$ exceeded the range of the ELISA reader, then the data file containing the $O.D._{450} - O.D._{490}$ readings was automatically called and the O.D. value for the corresponding well located and converted into $O.D._{405} - O.D._{490}$ units using the appropriate, empirically determined, conversion factor (5.3).

The values corresponding to experimental treatments, background controls and non-specific binding were then located by their position in the matrix. The experimental treatment values were corrected as required and the means of corrected experimental values computed (=reduced data). The details of this part of the program obviously varied from experiment to experiment, but by use of the appropriate experimental design,

the periodicity in the design could be used to construct simple loops and only very few lines of program code were required to describe the repetitive computational tasks.

The length of time in minutes from the beginning of the experiment to the time at which the reading was made and the reduced data were printed out, in neat tabulated form, and written back to the magnetic storage medium for further data processing. The files for subsequent readings were then located and the procedure repeated until the data from all readings had been processed.

The reduced data was then read into the computer and used to compute the rate of the ELISA reaction for each experimental treatment. This was done by computing the regression of $O.D._{405}-O.D._{490}$ values against time using the method of least squares outlined in section M.7.3. The processed data was finally printed out in the form of a list of the slope of each of these regressions (ELISA units) together with its 95% confidence limits.

By recording the data on magnetic media and using a microcomputer it was possible to process the data from 10 readings of a 96 well plate in about 30 minutes. The results represent the information extracted from 1000 individual measurements. It would take about two hours just to key (enter) this amount of data into a pocket calculator (allowing 5 seconds per entry) and probably at least an equal amount of time to compute, record intermediate results and to tabulate and write out the final results. It is therefore reasonable to estimate that, for the amounts of data which must be processed for rate ELISA measurements, electronic data processing is approximately 10 fold more efficient than hand calculation.

M.8 Sources of Reagents

Most chemical reagents were p.A. grade and were obtained from the company MERCK.

p-Nitrophenolphosphate, E.coli tRNA, 5S RNA and phage lambda DNA were obtained from BOEHRINGER

Pristan and DABA were obtained from EGA

BCIP was obtained from SERVA

M.9 Abbreviations and Acronyms

A/J	A mouse strain designation
Ab	Antibody
Ag or ag	Antigen
AS	Ammonium Sulphate
Balb/	A mouse strain designation (various sublines are indicated by letters after the slash)
BCIP	5-Bromo-4-chloro-3-Indolyl phosphate (p-toluidine salt)
BS	Borate Saline
BSA	Bovine Serum Albumin
C	Constant region (of IgG molecules)
C1, C1q	Components of the complement system
C57Bl6	A mouse strain designation
CBA	A mouse strain designation
CF	Complement fixation
C.L.	Confidence Limit
Con A	Concanavalin A
CNBr	Cyanogen Bromide
cpm	counts per minute
$D_{20,w}$	Diffusion constant
DABA	3, 5 Diaminobenzoic Acid
DAGB	Buffer for DEAE Affi-gel blue chromatography
DEAE	Diethylaminoethyl (group with which cellulose is derivatised to make an ion-exchanger)
DMEM	Dulbecco's Modification of Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid

DPBS	Dulbecco's Modification of Phosphate Buffered Saline (see p. 20)
dpm	disintigrations per minute (cpm corrected for counter efficiency)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunoadsorbent Assay
E.U.	ELISA Unit
Fab	Fragment of antibody (which contains antigen recognition site)
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
GAM	Goat Anti Mouse Antibody
GAR	Goat Anti Rabbit Antibody
H1	Histone 1
(H+L)	Heavy and Light chain (specificity of anti-antibody)
hnRNP	Ribonucleoprotein particle containing heterogeneous nuclear RNA
IEF	Isoelectric focusing
IEP	Isoelectric point
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
i.p.	intra peritoneal
IRMA	Immunoradiometric Assay
i.v.	intra venous
Kc	Designation of a Drosophila cell line
KBR	Buffer for Complement Binding Reaction (see M.2.5.1.2.1 p.55)
LKB	A commercial company
M	M Buffer (used IgM purification M.2.3.3.3 p.45)
MIP	Maximum Intensity Profile (see M.6.2, p.74)
MOPC	Designation of a class of mouse plasmocytomas
MPI	Max Planck Institute
MSA	Mouse Serum Albumin
M.W.	Molecular Weight

NS1	Designation mouse myeloma cell line from which hybridomas can be derived
O.D.	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PEG	Polyethyleneglycol
PMSF	Phenylmethylsulfonylfluoride
RAM	Rabbit Anti Mouse Antibody
RBC	Red blood cell
RIA	Radioimmunoassay
RNA	Ribonucleic Acid
RNP	Ribonucleicnucleoprotein particle
RPMI	Roswell Park Memorial Institute (Cell culture medium)
SB	Substrate Buffer (for ELISA, p.61)
s/c	subcutaneous
S.D.	Standard Deviation
S.E	Standard Error (of the mean)
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SRBC	Sheep Red Blood Cells
STU	Designation of mouse strain
TCA	Trichloracetic Acid
TE	Tris (10 mM pH 7.5), EDTA (1 mM) Buffer, standard storage buffer for nucleic acids
TEA	Triethanolamine (buffer substance)
TEN	TE buffer (see above) with 50 mM NaCl
TEPC	Designation of a class of mouse plasmocytomas
TMED	N'N'N'N' tetramethylethylenediamine
TRITC	Tetramethylrhodamineisothiocyanate
V, V_H, V_K	Variable region of Ig (heavy chain, kappa chain) polypeptides

RESULTS and DISCUSSION

FOREWORD

Choice of Antibodies and Antigens

The antibodies and antigens used in this study were chosen because they are, or were, being used by other members of the research group of which I am part.

The rationale for the selection of the hybridoma antibodies against Drosophila nuclear antigens is explained in SAUMWEBER et al., (1980). The mass production and purification of some of these antibodies was essential for, or greatly aided, the development of a number of immunological techniques: the "reverse RIA" technique for identifying antigens in RNA containing structures (RISAU et al., 1983); production of Fab fragments from serum derived antibodies (R.3.4.5); determination of absolute antibody amounts and testing the reliability of a quantitative assay for mouse antibody (R.8); precise definition the staining conditions for antigen comparison by double direct immunofluoresence (R.10).

Anti-H1 antisera and monospecific antibodies purified from them were originally produced (R2, R4) with a view to using immune electron-microscopy to localise H1 in chromatin with respect to DNA and the other histones (see RENZ et al., 1977, LÖFFLER 1978). This goal has not yet been achieved, possibly because of changes in the immunoreactivity of H1 upon chemical fixation (R.11.3). Nonetheless, the properties of this antibody-antigen system when H1 is complexed with nucleic acid may be of use in determining the function of H1 (R.11.2). The H1-anti H1 system provides an example of a polyclonal antibody directed against a nuclear component. In addition, the availability of pure H1 antigen has allowed the system to be used to characterise quantitative aspects of solid phase assay (R.7.3.4, R.7.4). Some idiosyncratic properties of the H1-anti-H1 system which were observed during production (R.2), purification (R.4) and assay (R.5) of anti-H1 antibodies are reported as being interesting in their own right.

RESULTS and DISCUSSION

PART I

METHODOLOGICAL ASPECTS OF ANTIBODY PRODUCTION, PURIFICATION AND MEASUREMENT

PART IA

Antiserum Production

R.1 Production of non-Balb/c origin hybridomas as ascites in Balb/c mice.

R.1.1 A consideration of the special Properties of Balb/c mice

B-cell hybridomas are antibody-producing cells with an unlimited proliferative capacity. They are produced by fusion of tumourous, drug-resistant cells of lymphatic origin with normal spleen cell lymphocytes. Almost all the tumourous cell lines used for murine hybridoma production are derivatives of plasmocytoma cells originally obtained from mice of the inbred Balb/c strain (for a recent review of hybridoma production see READING 1982 and for an extensive review of the properties of plasmocytomas see the classic work of POTTER 1972). The preponderance of Balb/c derived plasmocytomas is attributable to a property peculiar to these mice and not found in other inbred strains (POTTER et al., 1975). When Balb/c mice are injected intraperitoneally (i.p.) with a wide variety of different kinds of mineral oil, of which Pristan (2,6,10,14 tetramethylpentanedecane) is the most effective, they have a pronounced tendency to form plasma cell ascites tumors (i.e a tumor in the form of a suspension of lymphocyte-like cells termed plasmocytomas)(POTTER and BOYCE 1962, ANDERSON and POTTER 1969). Such tumors can readily be further passaged as ascites if they are injected into the peritoneum of Balb/c mice pretreated with Pristan i.p. (POTTER et al., 1972).

If the spleen cells used for a hybridoma fusion are of Balb/c origin, then the resulting hybridomas will carry only Balb/c histoincompatibility markers and can be grown, like their plasmocytoma progenitor, as ascites. The advantage of being able to do this is that the fluid in which the ascites cells grow accumulates large amounts of monoclonal antibody. Volumes of as much as 5 ml per mouse are not uncommon and in exceptional cases may reach 10 ml (my own observations). There

is some discrepancy in the recent literature about the amount of antibody which an ascitic fluid is likely to contain; GALFRÈ et al. (1979) estimate a concentration of 10 mg/ml in rat ascites but CROCE and KOPROWSKI (1982) mention a figure of 100 mg/ml for mice. The latter figure is almost certainly too high and even 10 mg/ml represents the upper end of the range, rather than being typical. In studies where the amount of antibody has actually been measured, using a plasmocytoma (FAHKRI 1970, FAHKRI and HOBBS 1970) between 5 and 8 mg/ml was found, but using hybridomas (RETH et al., 1978) the amount of protein secreted by individual clones was found to be very variable, and ranged between 0.3 mg/ml and 8 mg/ml. Concentrations as high as 3 mg/ml are however frequently encountered (see R.3.3.3, table 13) and this is an improvement by approximately a factor of 100 over the concentrations obtained from hybridoma cell culture medium.

At any rate, ascites fluid provides a convenient source of material for the preparative purification of antibody (see R.3). Furthermore, such ascites are easily established. Injection of 5.10^6-10^7 cell culture cells into pristan-primed Balb/c mice results in the production of 3-5 ml of ascites within 8 days of injection, with a success rate

(Nr. ascites producers) / (Nr. mice injected)

approaching 100% (data not shown, N. LUKÁCS personal communication, READING 1982).

R.1.2 Problems arising with "non-Balb/c" hybridomas

If the spleen cell fusion partner is not of Balb/c origin, a number of problems arises if growing the hybridomas in ascites form is attempted.

If Balb/c mice are used as recipients for the cells then the expected problems are mainly immunological. Firstly,

rejection problems are likely, because if the hybridomas bear other major histoincompatibility markers than Balb/c cells they will be destroyed by the immune system of their host. Secondly if the hybridomas could be induced to survive, but produced a strain-specific non-Balb/c allelic variant of one of the component immunoglobulin polypeptides, which are known to exist (MAGE et al., 1973, HERZENBERG and HERZENBERG 1978), then, if ascites growth were slow, the Balb/c host might produce antibodies against it.

If another inbred mouse strain is used as the spleen cell source, then immunological problems can be successfully avoided by growing the hybridomas in Balb/c x non-Balb/c hybrid mice, identical in "parentage" to the hybridomas themselves (OZATO et al., 1980, VOLLMERS et al., 1979). This does however require that the appropriate crosses be set up.

There is one further class of "non-Balb/c" hybridomas which must be considered. If the hybridomas are derived from spleen cells of a hybrid mouse (see e.g. EILAT et al., 1980, LEE et al., 1981) then, at least as far as their histoincompatibility properties are concerned, they will be triparental . There is no way to produce a triparental mouse using conventional breeding techniques!

Despite the problems mentioned above, there are situations in which it is neccessary to make non-Balb/c hybridomas. For instance, when one inbred mouse strain is used to produce antibodies against cell components of another inbred mouse strain (e.g. HÄMMERLING et al., 1978, VOLLMERS et al., 1979, OZATO et al., 1980) or when a particular strain spontaneously develops auto-antibodies against some tissue or cell component of interest e.g. RBC (haemolytic anemia)(PAGES and BUSSARD 1978); DNA or RNA (ANDREZEJEWSKI et al., 1980, EILAT et al., 1980, LERNER et al., 1981, LEE et al., 1981). Rat x mouse hybridomas are also useful for producing antibodies against murine antigens (GALFRÈ et al., 1977), but present a greater immunological problem. For such cases it would be

useful to have a generally applicable method for producing ascites. In the sections that follow I describe the development of a method that works for non-Balb/c hybridomas of murine origin.

This section would not be complete if I did not mention that one way to produce ascites from virtually any kind of hybridoma is to use athymic nude mice (FLANAGAN 1966, PANTELOURIS 1978, CROCE and KOPROWSKI 1982). This approach has been successfully employed to produce ascites from interspecific rat x mouse hybridomas (NOEMAN et al., 1982) and human hybridomas (CROCE and KOPROWSKI 1982). However, nude mice have the disadvantage that they are extremely susceptible to disease.

R.1.3 "Tübingen Mice"

The mice from which the hybridomas used in this study were manufactured (SAUMWEBER et al. 1980) were an inbred strain of white mice - "Tübingen mice" - which had been maintained in the animal house of Abteilung I of the MPI für Virusforschung for approximately 10 years by brother x sister matings. During this period the "Tübingen mice" had been selected for fecundity and vitality. They were designated (wrongly) Balb/CA, although their origin is obscure (K.KÖHLER, personal communication; K-U. HARTMANN, personal communication)

Attempts to produce ascites in these mice either from our hybridomas (SAUMWEBER pers. comm.), from the Plasmocytoma cell line P3-X63-Ag8 (the original Balb/c derived, antibody producing, tumorous fusion partner used for hybridoma production by KÖHLER and MILSTEIN) or from four other plasmocytoma cell lines obtained from Dr. POTTER met with almost no success. However, when Balb/cHan were obtained directly from the Zentral Institut für Versuchstiere in Hannover, injection of the plasmocytoma cell lines into unirradiated mice or of

two hybridoma lines (P11/3/6 and S5/7/7/6 - SAUMWEBER pers. comm.) into irradiated mice gave some success, although no exact statistics were kept at this stage. A success rate of about 50% was also achieved with the use Balb/cHan x "Tübingen mice" hybrids as ascites hosts (C.DREYER, personal communication) for another set of hybridomas derived from Tübingen mice (DREYER et al., 1981). The ease with which hybridomas can usually be propagated in Balb/c mice (see R.1.1), the at least partial success obtained using mice with a pedigreed Balb/c component compared to the almost complete failure using "Tübingen mice" suggested that "Tübingen mice" were not Balb/c. The non-uniform properties of DREYER'S F1 hybrids gave rise to the suspicion that the mice might not be genetically homogeneous.

Closer investigation of "Tübingen mice" yielded three pieces of evidence which argued overwhelmingly that this was indeed the case.

Firstly the average litter size of the "Tübingen mice" was >10, whereas the mean litter size of Balb/cJ subline from which the Balb/cHan subline is derived is 5.3 (ALTMAN and KATZ 1979).

Secondly "Tübingen mice" were noticeably larger than Balb/c mice derived from Hannover stock, although since the Balb/c strain is extensively inbred there should have been almost no variation in phenotype when the mice were kept under identical conditions.

Lastly histoincompatibility testing by Prof. Dr. D. GÖTZE of the Abteilung für Immungenetik, MPI für Biologie, Tübingen revealed that the mice failed to carry the typical immunological markers of Balb/c mice, but they did carry markers found on STU mice (SCHÄFER 1979; w34 haplotype, FIGUEROA et al., 1982) and on the Swiss White strain (q haplotype, KLEIN 1975). One mouse carried both markers, which means that the population of "Tübingen mice" contained mice heterozygous for H2 markers.

Table 7 shows that, although the population of "Tübingen mice" was inbred, heterozygotes could still have been found in it with a probability of 10% even if the initial hybridisation event were to have occurred 10 years previously and the breeding pairs had been selected by chance (assuming breeding pairs were replaced every 15 months). If either the initial cross were to have occured more recently, or if heterotic effects were to result in positive selection for heterozygous mice, then their frequency would be higher.

There are four major undesirable consequences of using a segregating non-Balb/c mouse line:

1) The hybridomas will be of non-Balb/c origin

2) The major histoincompatibility complex (H2) markers of hybridomas produced from different mice has a high probability of being different.

3) Some hybridomas would be triparental: i.e carry 3 sets of H2 markers.

4) Some mice would have novel genotypes and phenotypes. Apart from the H2 complex differences, inbred lines differ at other loci. Recombination in heterozygotes would create further variation, further increasing the variation between hybridomas obtained from different mice.

The 50% success rate obtained with the Balb/cHan x Tübingen hybrid mice could then be explained if the hybrid mice had either of two different histoincompatibility phenotypes (Balb/STU or Balb/q) and the hybridomas also had a 50% chance of having either phenotype, reflecting the distribution of phenotypes in the segregating population from both hybrids and hybridomas were produced. On a chance basis hybridoma/hybrid mice combinations which had a non-Balb/c parent in common would be expected to arise 50% of the time.

Generation	Genotypic Composition	Probability of heterozygote-containing litter in the next generation	Probability that mouse chosen at random will be heterozygous.
F_0	q/q x STU/STU (Swiss white) (STU strain)	-	-
F_1	q/STU	1.0	1.0
F_2	q/q STU/STU q/STU (with frequency) (0.25 0.25 0.5)	0.88	0.5
F_3	Frequency of litters with composition: all q/q 0.0625 all STU/STU 0.0625 q/q + q/STU (1:1) 0.25 STU/STU + q/STU (1:1) 0.25 as F_2 0.25 all q/STU 0.125	0.71	0.38
F_4	litter types as in F_3 but with increasing frequency of all homozygote litters	0.59	0.31
F_5		0.48	0.25
F_6		0.39	0.20
F_7		0.32	0.17
F_8		0.26	0.14
F_9		0.21	0.11

Table 7 **Probability of a breeding colony producing mice heterozygous for a single locus calculated for 9 generations of brother x sister matings.**

Starting from an initial cross between mice from two inbred strains homozygous at the H2 locus for the q and STU haplotypes, column 3 shows the frequency and possible genetic composition of litters arising from brother sister pairs chosen at random from litters chosen at random from the population. Column 4 lists the probability that heterozygotes will continue to be carried in the population and the last column the probability of an individual mouse being a heterozygote.

The original "Tübingen mouse" line has since been destroyed. The problem remained of finding a reliable way to produce ascites tumors from hybridomas derived from these mice. Luckily, as the following section reveals, the remedy is simple.

R.1.4 Non-Balb/c hybridomas can be established as ascites in irradiated Balb/c mice

Balb/cHan mice were chosen as hosts for the Tübingen mouse hybridomas, because of their genotypic and phenotypic uniformity and because of their special properties with respect to the plasmocytoma induction (see R.1.1). Providing that histoincompatibility characteristics were not lost following fusion, the hybridomas should always have between 33% and 50% of their H2 markers (from the tumourous fusion partner) in common with these mice and a particular hybridoma should therefore produce a predictable response from the mice. The mice were irradiated with 360 Rad gamma irradiation to cripple their immune system. Balb/c mice are particularly radiation sensitive (STAATS 1980) so it is not possible to use irradiation doses which are customarily used to eliminate the immune system of other strains entirely. It was however hoped that the hybridoma cells would grow quickly enough in the face of a weakened immune challenge to be able to give rise to a viable ascites before the immune system recovered.

Table 8 shows that ascites of non-Balb/c hybridomas can be produced with high frequency in irradiated Balb/c mice. The overall success rate (ascites produced / mice injected) was 92%. 58% of the hybridomas gave rise to ascites in all mice injected and no hybridoma failed to give rise to at least one ascites. All the ascites which were produced could be propagated by serial passage (see R.1.4).

Close examination of the data in table 8 reveals considerable differences between individual hybridomas. The time before the appearance of the first ascites varies between 9 d

Line Nr.	Initial Innoculum x 10^{-6}	Name of Hybridoma	Ascites/ Injected	Time to first ascites (days)	Time to last ascites (days)
		ASCITES TUMORS ESTABLISHED WITH 100% SUCCESS RATE			
1	1.0	Q18/5/4	6/6	10	10
2	8.0	T50/7/11	5/5	9	12
3	7.0	Aj1/2/2	5/5	10	14
4	3.6	V4/9/6/1	5/5	13	14
5	7.6	U25/1/9/1	5/5	10	16
6	9.0	P3/X63-Ag8*	3/3	20	24
7	7.2	W2/8/1/3	5/5	20	24
8	8.0	P12/8/6	6/6	15	25
9	10.0	W12/10/6	5/5	16	33
10	4.5	X4/8/6	6/6	15	58
11	?	Z4/10/9	6/6	14	66
12	?	T37/4/17/4	5/5	16	67
		ASCITES TUMORS ESTABLISHED WITH LESS THAN 100% SUCCESS RATE			
13	7.6	T29/2/2	4/5	11	14
14	7.6	T40/7/7	3/4	12	18
15	6.5	T7/3/3/4	4/6	22	93
16	9.0	Ah6/5/9	1/3	49	-
17	9.0	P3/X63-Ag8* (unirradiated)	1/3	56	-
		No figures available, established by H.SAUMWEBER "with difficulty"			
18		P11/3/6			
19		P11/6/3			
20		S5/7/7/6			

Table 8 **Establishment from cell culture of non-Balb/c origin hybridomas as ascites in irradiated Balb/c mice.**
? not recorded. * plasmocytoma not hybridoma.

- -

for T50/7/11, (line 2 in table) and 49 d, i.e. 7 weeks, for Ah6/5/9 (line 16 in table). The time **period** over which ascites are produced from the same initial innoculum also varies greatly, between 1 d for Q18/5/4 (line 1 in table 8) and 71 d, i.e. more than 2 months, for T7/3/3/4 (line 15 in table 8). Given this variation, to obtain ascites growth of an untried hybridoma, within a reasonable period of time, several (at least 3) mice must be innoculated initially.

The reasons for the observed variation are unknown. There is variation between the hybridomas derived from the cells of a single mouse e.g. the various hybridomas from fusion "T" (lines 2, 12, 13, 14 and 15 in table 8), so at least some of the variation may be ascribable to intrinsic properties of the hybridomas themselves, rather than their genetic origin. The Balb/cHan mice used as ascites hosts were not matched closely for age or sex. However, examination of the records shows no discernible systematic effect of either of these factors.

Control experiments with unirradiated mice were not made for all of the hybridomas. It was felt that, in view of the known histoincompatibility properties of the "Tübingen mice" from which the hybridomas arose and of the poor success rates reported in section R.1.2 that the sacrifice of these mice could not be justified in terms of the the goal to be achieved. The possibility therefore exists that some of the hybridomas might have grown in unirradiated mice. Even if this were the case, it would not alter the conclusion that the ploy of irradiation and the use of Balb/c mice as hosts provides a single set of conditions under which many Tübingen hybridoma lines can be grown.

One further point of note is that the myeloma line P3-X63-Ag8 although itself originally an ascites of purely Balb/c origin, grew significantly better in irradiated than in unirradiated mice (compare lines 6 and 17, table 8) mice. There are two possible explanations for this behaviour. One is that it might have been caused by a histoincompatibility difference between the P3 cells, which are derived from the Balb/cAnN subline (POTTER et al., 1965; HORIBATA and HARRIS 1970) and the Balb/cHan mice which come from the Balb/cJ subline. The /cJ and /cAnN sublines became separated 50 years ago after the first 26 generations of inbreeding (ALTMAN and KATZ 1979). The mice of the two sublines differ in that the males of the Balb/cJ subline are more agressive (STAATS 1980) and in the allele carried at H-2^{Qa-2} locus (DEMANT and ROOS

1982: these authors in fact used the Balb/cBy and Balb/cHe sublines, but these are very closely related to Balb/cAnN and Balb/cJ respectively). The other possibility is that the P3 cell line has become sufficiently altered (? as a result of somatic mutation?) during the 11 years it has been maintained in cell culture to be rejected as foreign in unirradiated recipient mice.

R.1.5 Adaptation of hybridoma lines to ascites growth and growth in unirradiated mice

Table 9 shows that on serial passage the time taken for an ascites to develop remains approximately constant for those hybridomas the duration of whose passage was initially short (< 10 days), whereas for those lines which initially took longer (>11 days) the duration tends to diminish to 10 days or less (despite occasional aberrations such as that shown by hybridoma T37/3/3/4 during the fourth passage). This suggests that whatever the reason for initial differences in growth rate, the hybridomas tend to adapt (i.e. selection takes place) to growth in mice.

Hybridoma name	Duration of passage (days)				
	Passage Number				
	1	2	3	4	5
Aj1/2/2	10	10(0.3)	8(0.3)	5(0.3)	
U25/1/9/1	10	7(0.2)	8(0.1)	8*(0.2)	
T29/2/2	11	8(0.2)	12(0.1)	9(0.1)	
T37/4/17/4	16	14(0.2)	16(0.1)	50(0.1)	8(0.1)
W2/8/1/3	20	12(0.2)	6(0.1)	7(0.1)	
T7/3/3/4	22	15(0.2)	10(0.2)	12(0.1)	
Ah6/5/9	49	12(0.3)	14(0.2)	10(0.3)	

Table 9 **Adaptation of hybridomas to growth as ascites in mice.** The data shows the passage time of the fastest growing ascites in each passage. All mice were irradiated except *. Number in parentheses is amount of ascites used to start passage in ml.

Attempts were therefore made to see whether growth could be achieved in unirradiated mice. These attempts were successful (see e.g table 10), and all the hybridomas shown in table 8 can now be grown in unirradiated mice. This observation suggests that histoincompatibility barriers can be overcome by selection. It would be interesting to monitor directly the phenotypic and genotypic H2 status of these cells.

No systematic investigation was made of how best to establish growth in **unirradiated** mice. The initial attempts were made after 7 passages in irradiated mice, and most of the hybridomas listed in table 8 were adapted after 3 or 4 passages. Growth of one line (Aj1/2/2) was established on the second passage. Whether all lines can be adapted this early remains to be investigated.

R.1.6 Long Term Stability of Ascites

More than half of the hybridomas listed in table 8 were subjected to 8 or 9 serial passages without any sign of instability and since this sufficed to produce enough material for preparative antibody preparation, the ascites adapted lines were then stored frozen.

However, 5 of the ascites adapted hybridomas (P11, P12, S5, T29 and T37) showed "crises", in which either antibody production or ascites tumor growth ceased. One example has already been shown in table 9, in which the fourth passage of hybridoma T37/4/17/4 took 50 days (i.e. 7 weeks) in contrast to the 16 days and 8 days taken by the preceding and subsequent passages. Another example, in which the P11/3/6 hybridoma was affected, is shown in table 10. After 16 passages in which liquid ascites had been regularly produced, the growth pattern in 3 mice from 2 parallel lines (started 3 passages earlier) suddenly altered. In one mouse, an ascites developed but antibody activity was absent. In the other two

Passage Nr.	Innoculum (ml)	LINE 1 Passage duration (d)	Yield (ml)	Innoculum (ml)	LINE 2 Passage duration (d)	Yield (ml)
2*	?	13	3.1			
3*	0.3	10	3.1			
4*	0.3	11	4.0			
5*	0.15	13	3.2			
6*	0.1	11	3.3			
7*	0.1	9	3.8			
8	0.2	9	1.6			
9	0.1	9	2.0			
10	0.1	7	1.0			
11	0.1	11	2.3			
12	0.1	8	2.0			
13	0.2	10	3.0			
14	0.2	8	3.5	0.2 (cells of line 2 stored frozen for) (13 days, then thawed)	10	3.0
15	0.2	8	3.5	0.5*	12	2.1
16	0.15	8	2.5	0.3	10	4.0
17	0.1	41	SOLID	0.3	14	3.5(NEGATIVE)
17a	(from LINE 1 SOLID TUMOR)			0.3	20	SOLID (not tested)
18	0.1	9	2.5			
18a	0.1	11	2.0			
18b	0.1	12	0.5			

Taken into culture and cloned from passage 17. Grows very poorly despite macrophage addition to culture. Cloning efficiency $<10^{-6}$. 1 clone obtained.
Returned to ascites culture after 2 months cell culture.

19*	4.10^6 cells	13	2.0
20	0.3	9	3.5
(from frozen stock after 17 months storage)			
22-25	0.3	7-10	0.5-5.0

Table 10 **Unstable phenotype of ascites-adapted hybridoma P11/3/6.** An alphabetic character following the passage number indicates results on replicate mice. * indicates these mice were irradiated. Parallel lines were maintained from the two mice innoculated from passage 13.

- -

mice tumor growth took a longer time than in previous passages and a solid tumor developed. One of these solid tumors was nonetheless able to give rise to normal ascites which could be further passaged. Although the events occurred

during the same passage, they were chronologically separated, because one of the lines had been stored frozen. This and the fact that other hybridoma lines were behaving normally at the time these events occurred make trivial explanations, such as a faulty batch of Pristan unlikely.

The mice used in these experiments were offspring of several pairs of mice which had been obtained directly from Hannover and were assumed to be pure bred. Unfortunately one of two sibs from the progeny of one of these breeding pairs was subsequently found to lack the appropriate Balb/c H-2D^{d} histoincompatibility marker, even though no marker from a foreign strain could be identified (I am indebted to Dr. Figueroa, MPI für Immungenetik, Tübingen for carrying out the test). This sib also sired abnormally large litters. This suggests a genetic anomaly in the original shipment and makes it possible that some of the observed "crises" may be explicable on the trivial ground of immune rejection. However since "crises" occurred in less than 1 in 20 of the mice the frequency of such rogue mice must have been low. The probability of choosing 3 such mice at random is in the order of .01%. A more likely explanation would be that the cause of the three crises in the same passage may have lain in the hybridoma cells rather than their hosts.

If a single mutational event (possibly a chromosomal rearrangement) occurred to one of the P11 cells sometime before or during passage 13, it is possible to calculate that the progeny of this cell would not begin to dominate the population until 3 passages later. Assuming that the ascites growth rate is similar to that found for a number of plasmocytomas (POTTER 1972, FAKHRI 1970) approx. 10^{5} cells growing to 10^{8} cells in about 10 days, a single cell mutating to give daughters with a 10% shorter generation time than the rest of the population would be required to take over the population so quickly. If the growth rate advantage were lower then the mutational event would have to occur earlier.

The appearance of 1 altered growth rate mutant in approx.

130 cell generations in populations of the size indicated requires a mutation rate of 10^{-7}/cell/generation. This does not seem excessive. Plasmocytomas with altered phenotypic characteristics such as cessation or reduction of antibody production have been observed after multiple passage (POTTER 1972, FAHKRI and HOBBS 1970). Alterations in antibody production at a frequency of about 10^{-7}/cell/generation have also been observed in plasmocytoma and hybridoma cell growing in vitro: loss of production of one or both chains (KEARNEY et al., 1979, MILSTEIN et al., 1977, KÖHLER 1980, KÖHLER and SCHULMAN 1980), altered antibody class (LISEGANG et al. 1978, FRANCUS and BIRSHTEIN 1978, FRANCUS et al. 1978), changed V region expression (LAVIN and SIBLY 1981), deletion of amino acid sequences (KÖHLER and SCHULMAN 1980, MILSTEIN et al., 1976) and secretory defects (KÖHLER et al., 1976) have all been described. In view of the pluripotent nature of the B-cell lineage and the frequent gene rearrangements involved in producing its various branches during normal development (WABL and STEINBERG 1982, COUHTIHNO et al. 1982), it is perhaps the stability rather than the lability of the ascites adapted hybridomas which is suprising.

This section has attempted to make clear both on the basis of my own observations, and by reference to the literature, that there is a small but significant intrinsic instability associated with hybridoma cell lines, which can become apparent upon multiple passage. This kind of instability is quantitatively different to and should be distinguished from the instability which occurs during the initial stages of hybridoma cloning. The observations described in the literature suggest that this property is also observed in established "pure" Balb/c hybridomas. Properly controlled, this instability is not a serious problem: perseverent monitoring allowed all the cell lines listed in table 8 to be passaged further. However, if the production of antibody by ascites cells is not stringently controlled loss of cell lines could occur, or the rate of production of

ascites could be seriously curtailed. A practical lesson from the observations reported in this chapter is that cells from more than one passage should be stored frozen to ensure the survival of ascites-adapted lines. If instability proves to be a continuing problem then the cells can also be returned to cell culture and recloned as shown in table 10.

R.1.7 A successful recipe for long term storage of hybridoma cells

Using the procedures described in M.2.1.6 and M.2.1.7, 20 ascites-adapted hybridomas have been revived after frozen storage. 3 of them have been revived more than once. The longest storage period was 21 months. Only one failure has been recorded to date.

R.2 Polyclonal rabbit anti-H1 antisera: immunisation schedules

There is a concensus of opinion in the literature that pure histones are poor immunogens (HEKMAN and SLUYSER 1973, STOLLAR 1978, BUSTIN 1978). However SANDBERG et al., (1967) and STOLLAR and WARD (1970) have shown that complexes of purified core histones with nucleic acids are effective antigens. Antibodies to H1 have also been produced using complexes of this type (BUSTIN and STOLLAR 1972, 1973a), but there have also been two reports that antibodies to H1 can be produced without using nucleic acids (POTHIER et al., 1975, MIHALAKIS et al., 1976). An attempt was therefore made to raise antibodies against H1 in both complexed and uncomplexed form. There were two main reasons for doing this. Firstly, to test whether H1-nucleic acid complexes really are more immunogenic than H1 alone. Secondly, since complexing an antigen could mask or alter antigenic sites, if antibodies could be raised by each of the two routes it would be of interest to compare their properties. In addition, the influence of different immunisation sites, adjuvants, and frequency of injection was was compared, to see whether it might be possible to find an immunisation protocol particularly suited to raising anti-H1 antibodies.

The titres obtained using various immunisation schedules on 9 outbred rabbits are summarised in table 11. There are no obvious correlations between the various routes of injection, or adjuvant formulations, and subsequent antibody titre, except perhaps that, with the exception of rabbit 141, there is a large rise in titre following high (500 µg) i.v. doses. There is clearly variation between rabbits: rabbits 141 and 147 were subjected to identical immunisation protocols but gave a 10 fold difference in response.

The response of rabbit 142 shows that although it is custom to use Freund's complete adjuvant, this is not an absolute requirement for obtaining an immune response with H1.

In two of the rabbits (138, 145) it took three immunisations (and more than three months) and in another (150) four immunisations before a measureable antibody titre developed. Despite the delay rabbit 138 went on to produce the antibody titre with the second highest ranking. The response of rabbits 138 and 150 shows that it is not neccessary to complex H1 with nucleic acids to obtain an immune response nor even, in the case of rabbit 138, an elevated antibody titre.

One rabbit (147) out of the 9 became obviously hyperimmune, developing and maintaining a titre 2 to 3 times that of the other rabbits. On continued immunisation the titres of all rabbits tended to fall but, by using complete Freund's adjuvant, by performing consecutive immunisations within a short period of time (seven days) and by using a high (400 µg) i.v. dose (details cf. M.2.2.3) it was possible to raise titres 5-10 fold (see footnotes, table 11) to >200 µg/ml anti-H1. Since there is some risk that the animals may succumb to anaphylactic shock as a result of this procedure it was only used before the animals were slaughtered.

The dosages of H1 used (100-400 µg) lie at the lower end of the range used by previous investigators (BUSTIN 1978), but are obviously adequate, although the response to i.v. injection may be dose dependent (see above). Indeed, the ability to produce a hyperimmune response (rabbit 147) by using dosages in this range suggests that even lower dosages might still be effective. The data in table 11 is inadequate to determine what a minimum dose might be.

After this work was completed, BUSTIN (1978) compared the immunisation protocols used by 12 different investigators to raise anti-H1 antibodies and come to the conclusion that the response is indifferent to the exact protocol. My results do not contradict this view. There is a practical consequence which follows from these observations. Single emulsions with adjuvant are extremely viscous and difficult to inject. Double emulsions (HERBERT 1978), although more difficult to prepare, are more easily injected. Subcutaneous injections are easier

Rabbit Nr.	Coat Colour	Antigen	Immunisation Protocol = Letters Dose H1(µg)= Numbers (Immunisation Nr.) (1)	(2)	(3)	(4)	Antibody Titre µg/ml (Bleeding Nr.) (1)	(2)	(3)	(4)
138	Col.	H1 only	CEM 400	IES 400	IEV 100	IEV 450	-	-	6	96[1]
139	"	H1+tRNA	IEM 400	IES 400	IEV 450	XXX	-	-	-	X
140	"	"	CDS 400	IEV 400	IDV 500	IEV 200	-	96	48	12
141	"	"	CEM 400	IES 400	IEV 450	IDV 100	-	12	24	24
142	"	"	IDS 400	IES 400	IEV 100	IDV 500	-	-	N.T.	24[2]
145	Whi.	H1+tRNA	IEM 400	IES 400	IEV 100	--V 500	-	-	12	6
146	"	"	CDS 400	IES 400	IEF 450	XXX	-	-	-	X
147	"	"	CEM 400	IES 400	IEV 500	IDV 100	-	48	240	240[3]
150	"	H1 only	CEM 400	IDS 400	IEV 100	IDV 100	-	-	-	6

Footnotes: titres of further immunisations shown as titre (Bleed Nr.)
[1] remained same after 3 further immunisations
[2] 24(5), 6(11), 170(14=final, special protocol see text)
[3] 191(6), 136(8), 67(9), 380(14=final, special protocol see text)

Key to table 11

Column 2 Col.=Coloured Whi.=White

In columns 4-7 a three letter code summarises combinations of immunisation variables.

First letter, Adjuvant: I=Incomplete, C= Freund's complete, -=none
Second letter, Emulsion: E=Simple, D=Double -=none
Third letter, injection site: M=intramuscular S=subcutaneous V=intravenous
XXX=Slaughtered

Columns 8-11 "-" = < 6 µg/ml, X = Slaughtered, N.T. = not tested

Table 11 **Immunisation schedules and anti-histone 1 immune response of 9 outbred rabbits.**

Although more than one bleeding was sometimes made after an immunisation all the values in this table correspond to the first bleeding made after the correspondingly numbered immunisation. Antibody titres before bleeding Nr. 6 were measured by complement fixation (CF, R.6), later ones by IRMA (R.7). The second and third bleedings of rabbit 147 were tested in parallel in an IRMA assay calibrated with affinity purified antibody 402 (cf. R.7.3.4.1). CF titres have been converted to absolute concentrations on the basis of this calibration. The absolute amount of H1-specific antibody recovered from the serum of rabbit 147 indicates that these figures cannot be in error by more than a factor of 2. Except for the final immunisation (see text), the immunisation protocol after immunisation Nr. 4 was IDS using 100 µg H1.

to make than intramuscular ones, and probably cause the animals less suffering. Since it should not compromise the immune response the combination of double emulsion and subcutaneous injection can therefore be recommended for future immunisation programs.

Three further recommendations can be made on the basis of the data in table 11. Firstly, because of variation between rabbits and because of the long response times large numbers of rabbits should be used. Secondly, their serum titres should be monitored regularly. Finally, patience should be exercised if antibody against H1 is to be successfully produced in rabbits.

RESULTS AND DISCUSSION

PART IB

Antibody Purification and Characterisation

R.3 Mouse monoclonal antibodies

R.3.1 Why purify antibodies?

Apart from specific antibody, ascites sera also contain small amounts of other antibodies with other specificities, numerous other proteins, and a variety of other biologically active components (e.g. steroid hormones). In addition, the presence of pristan in the ascites sera can cause the formation of an oily emulsion. Furthermore, these components may vary from one ascites serum to another. To a lesser extent, some of these properties (e.g. the presence of other proteins) also apply to hybridoma supernatants used as a source of specific antibody.

For the indirect staining of fixed histological preparations, from which only qualitative information is required, presence of these other components in the antibody containing solution may not be important. The same is true of other qualitative tests for the presence or absence of some factor (e.g. a pathogen) in biological materials. In some cases, the presence of a large amount of other proteins in the serum may even be advantageous if it suppresses non-specific binding of specific antibody. Equally, however, there may be cases in which non-specific antibodies or other proteins themselves cause non-specific staining of, or signal generation from, biological materials. Use of purified antibody would clearly allow these situations to be avoided.

If more precise information from antibody-antigen reactions is required, undefined serum components are still more likely to cause problems. Five examples of the kinds of problem that can arise are outlined below.

1) If quantitative information is required, it is often neccessary to know the amount of monoclonal antibody being used in the system (see R.7.4). However, it is difficult to estimate amounts of antibody accurately in the presence of

other proteins (see M.1.1 and R.8).

2) If functional systems are to be investigated (e.g. by inhibiting the function of some cell surface component or an enzymatic reaction in a subcellular fraction), non-immune components of the ascites serum may also influence the functioning of the system.

3) If antibody is to be derivatised, labelled fluorescently (R.3.4.4), radioactively (R.7.4.5.) or coupled to enzyme, use of ascites serum may make it difficult or impossible to determine antibody specific activity, because of other derivatised proteins. In addition, other components may compete for derivatisation reagents, rendering derivatisations either very inefficient or difficult to standardise.

4) If antibody is to be modified by proteolytic digestion, either by production of Fab fragments to eliminate bivalent binding, or to $(Fab')_2$ fragments to eliminate unwanted effects of the F_c portion of the molecule, serum may cause problems, because mouse serum contains protease inhibitors. These are able to completely block the digestion of IgG (R.3.4.5). It is therefore neccessary to remove these components to obtain a successful proteolytic digest.

5) Serum also contains proteases. These may cause degradation of some monoclonal antibodies following prolonged storage (R.3.4.5). Alternatively, such proteases may interfere with various steps of purification procedures (R.3.3.2).

These problems are not hypothetical. They were either encountered (or avoided) by myself, in the sections of the thesis to which I have referred above, or they have been encountered by other investigators.

There are thus good reasons for wanting to have (a) reliable and efficient procedure(s) to purify monoclonal antibodies, especially when they are derived from ascites serum. However, a number of difficulties must be overcome before antibodies sufficiently purified to avoid all the problems described above can be obtained. Some of the

theoretical and practical reasons for these difficulties are outlined in chapter R.3.2 below, but see also section R.3.3.1 and the introduction to chapter R.3.4. The methods I have adopted to overcome these difficulties and purify monoclonal antibodies are described in chapters R.3.3 and R.3.4. Finally, chapter R.3.5 documents some of the properties of the antibodies produced by hybridomas which have been obtained in pure form. The examples of R.3.5 indicate that, as expected on theoretical grounds (R.3.4.1), even purified monoclonal antibodies cannot be considered to have uniform properties, and that this must be taken into account when working with them.

R.3.2 Problems which may be associated with the purification of mouse monoclonal antibodies

R.3.2.1 A priori considerations

The antibody repertoire of most mammals is estimated to be on the order of 10^7 (see WILLIAMSON 1976 for review of the evidence upon which this estimate is based). The molecular basis for this diversity is the pairwise combinatorial association between members of two large sets (10^3) of polypeptides (heavy and light chains). The members of each set differ from one another in amino acid sequence in the so-called variable region at their N-terminal ends. A number of mechanisms is responsible for the variation in the amino acid sequence of the variable region of both the light and of the heavy chains (for a recent reviews on how diversity is generated see HONJO 1983, TONEGAWA 1983).

Amino acid sequence differences would be expected a priori to give rise to differences in the physical properties between individual (monoclonal) antibodies, in addition to the inescapable differences in antigen binding properties. A measurable example of such differences can be seen in fig. 15, which illustrates the range of isoelectric points which can be

seen in a **selected** population of antibodies produced by a polyclonal response. Fig. 11 shows the measured variation of some purified monoclonal antibodies. The normal range of isoelectric points in mice is known to extend from pH 4 to pH 8 (see WILLIAMSON 1978 and references therein). Some physical properties which might be expected to vary between antibodies are solubility, ease of denaturation and non-specific binding to charged or hydrophobic surfaces. The dependence of such properties on variables such as ionic strength, pH and temperature would also be expected to vary from antibody to antibody. Some of this variation would result from differences in the extent and distribution of charge on the molecule and would be reflected to some extent in its IEP, but it is easy to think of charge independent mechanisms by which the the physical properties of the antibodies could be affected. A very subtle example of such properties is provided by a related family of monoclonal antibodies against the arsenate hapten, whose ability to be recognised by anti-idiotypic antibodies is quantitatively affected by variation in V_H, but only in the context of a particular V_K sequence on the light chain (CAPRA et al. 1982). High resolution X-ray diffraction analysis of antibodies (SILVERTON et al., 1977) has only just begun to be applied systematically to show the structural basis of such properties (for a review see DAVIES and METZGER 1983).

Almost continuous variation of antibody properties can be caused by V-region variability. In addition, a grosser level of heterogeneity in physical properties is provided by differences between the constant regions of molecules belonging to different classes and, in those animals which possess them (e.g. mouse and man but not rabbit), at the less pronounced level of subclass. The properties of either the V or the C part of an Ig molecule might also vary as a result of V-C interactions.

R.3.2.2 Some remarks on published purification procedures

Published purification methods for antibodies have until recently mainly been developed for purifying polyclonal antibodies (FAHEY and TERRY 1978, JATON et al., 1979, HURN and CHANTLER 1980, JOHNSTONE and THORPE 1982). Most involve an ion exchange chromatography step and rely upon the average properties of Ig molecules. Losses of specific antibody resulting from the aberrant behaviour of a few individual species within the total population could usually be tolerated, although yields from the ion exchange columns are not neccessarily maximal (see section R.4.3 for an extreme example). These columns will also lead to selective loss of acidic (negatively charged) antibodies; this is probably the reason for the relative paucity of acidic antibodies in the 402 anti-H1 preparation which can be observed in fig. 15 by comparing the focussing pattern with that of the 147 preparation (prepared by affinity chromatography without a prior ion-exchange chromatography step). In contrast monoclonal antibodies are uniform, and as a result some purification methods might be expected to fail entirely if the antibody being purified were to have the "wrong" pI.

A further minor problem which faces anyone wishing to purify mouse antibodies is that there is little detailed information about their purification which might be useful as a guide to developing methods. Little work has been carried out with polyclonal sera because of the miniscule amounts of sera available from individual animals. What little information is available suggests that their properties differ slightly from those of other species: "DEAE fractionation does not work well...(with mouse antibodies)". (JOHNSTONE and THORPE 1982).

In contrast, prior to the advent of hybridoma technology, many monoclonal antibodies had been isolated from myeloma ascites, using methods established for other species (POTTER et al., 1965, SVASTI and MILSTEIN 1972a). The antigens that

these mouse antibodies recognised were in most cases unknown, at least at the time of their isolation, and they were used mainly for sequence studies (POTTER et al., 1965, SVASTI and MILSTEIN 1972b, see POTTER 1978 for review) for which purity, rather than yield or functionality was a major criterion. More recently, after the completion of the experimental part of this work, PARHAM et al. (1982), reported the preparation of 7 highly pure, active, mouse monoclonal antibodies of the IgG1 subclass using ion exchange (DEAE) chromatography and the conditions they used will be a useful guide for future purifications. BRUCK et al., (1982) have described suitable conditions for the purification of monoclonal antibodies of the IgG1 and IgG2 subclasses on DEAE affi-gel blue.

The "standard" method described in the following section was designed to be as universal as possible, avoiding the pitfalls described above. Two simple steps suffice to provide IgG preparations whose purity is in the order of 80-90%. If further purification was required the methods desribed in section R.3.3 were used, but these had to be customised at least for the subclass of the antibody involved.

R.3.3 A "standard" method for purification of mouse monoclonal IgG

The "standard" procedure consists of producing a fraction containing the high molecular weight proteins in ascites serum by a series of precipitations with ammonium sulphate, followed by chromatography on a Sephacryl S300 column to separate native IgG (M.W. 150,000) both from a multiplicity of proteins of higher molecular weight and from one major protein, mouse serum albumin (MSA), of lower molecular weight (M.W. 65,000). Fig. 3A documents the $O.D._{280}$ profiles of S300 column eluates of the sera of 3 hybridoma ascites sera purified in this way. As explained in the figure legend the relative heights of the peaks in these profiles cannot be used directly as a measure of the amount of protein in the

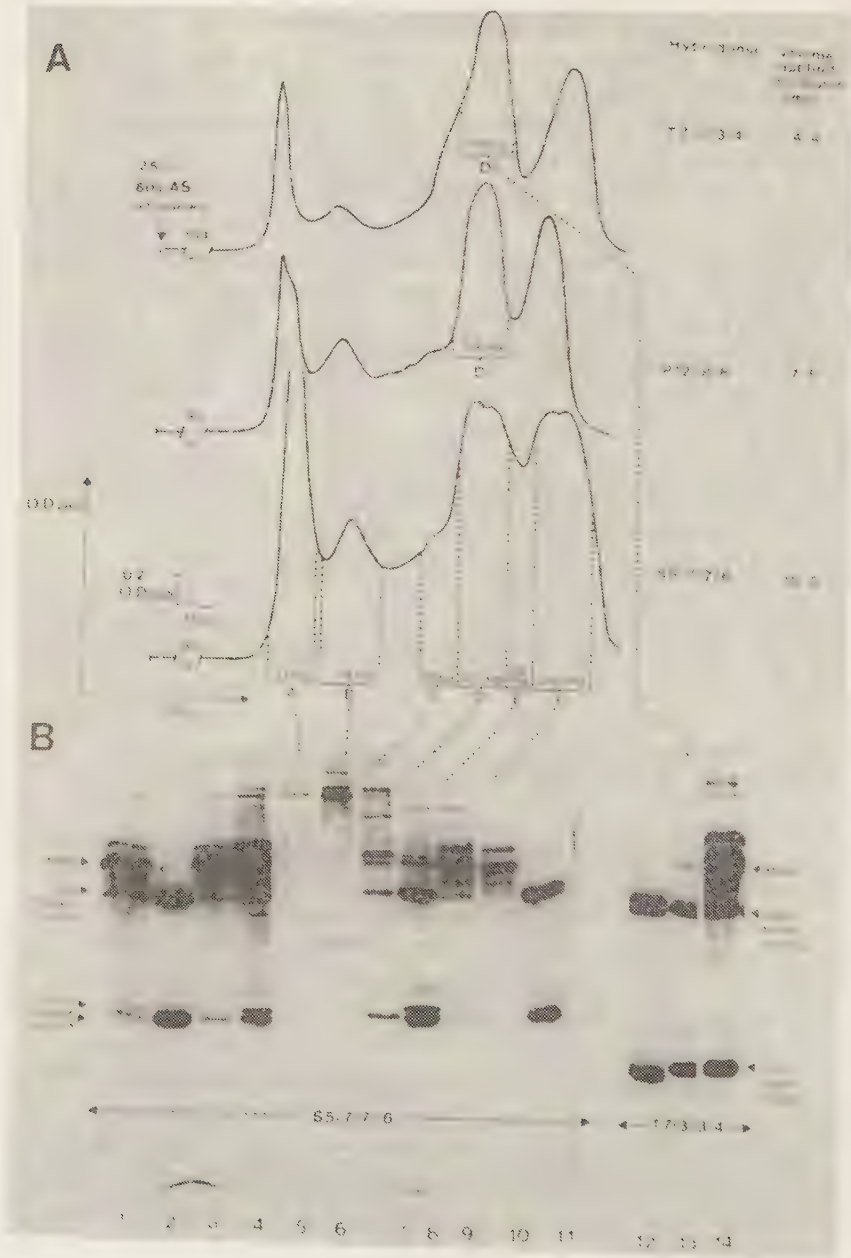
A
B
B
B

Fig. 3 **Purification of Mouse IgG from ascites sera on a Sephacryl S300 chromatography column.**

Part A. **Elution profiles of 25%-60% AS "cut" from ascites sera of 3 different hybridomas.** The hybridoma name and the volume applied to the column are indicated on the right hand side of the figure. The original volumes of sera from which the AS "cuts" were obtained are listed in table 13. The same column (76 cm x 4.5 cm diam., 1.2L volume) was used to chromatograph all three sera. The designations and protein content (Bradford method) of the pools of fractions whose analysis is shown in part B are shown next to the curly brackets marking their position in the eluate. The comparative heights of the two final peaks (IgG, pool D and MSA, pool F) give a false impression of the relative abundance of each protein. IgG absorbs more than twice as strongly as MSA at 280 nm when solutions with equal w/v concentrations are compared (M.1.1.1, table 3). Thus the mass of MSA exceeds that of IgG in all three preparations. In addition, the flow through spectrophotometer grossly underestimates the $O.D._{280}$ of solutions with a true O.D. >1.5., so peak heights exceeding this value are not directly comparable. The high "$O.D._{280}$" peak of the pool A material is mainly attributable to light scattering (hence the low protein content). The profiles have been aligned with respect to the IgG peak (non-alignment of the other peaks is attributable to differences in flow rate), and are plotted as a function of time, rather than fraction number, because the first peak eluted contained surfactant material which caused the fraction size to alter (because fraction size was measured by counting drops). IgG identity was confirmed by semi-quantitative Ouchterlony analysis with anti-mouse IgG.

Part B **Polypeptide composition of fractions analysed by SDS gel electrophoresis in 12.5% polyacrylamide gels.** Lanes 1-11 fractionation of S5/7/7/6 ascites. The source of the samples and the amount of protein applied (correction for different protein composition as in Table 12) were (amount of protein in µg in brackets): lane 1 60% AS pellet (29), lane 2 Mouse IgG standard (20), lane 3 clarified ascites (23), lane 4 28%-60% AS fraction (37); lanes 5-10 pooled fractions from S300 separation: dashed lines indicate source in panel A: 5(5), 6(8), 7(10), 8(23), 9(11), 10(11);lane 11, mouse IgG standard (20). Lanes 12-14 Fractionation of T7/3/3/4 ascites. lane 12, Mouse IgG standard (20), lane 13 IgG pool (20) from S300 column, dashed vertical line shows corresponding part of absorbance profile, lane 14 clarified ascites fluid (100). Lanes 1-11 and 12-14 are from two different electrophoretograms; the position of the bands for MSA and for the heavy and light chains of IgG are indicated on each.

fractions which give rise to them. Fig. 3B shows the polypeptide composition of two of these ascites sera (lanes 3 and 15), of ammonium sulphate precipitates from one of them (lanes 1 and 4) and of pooled fractions obtained from one of the columns (lanes 5 - 10). It is clear from fig. 3 that the purification procedure is reproducible and produces a fraction

which contains IgG that is between 70% (fig. 3B, lane 8) and 90% (fig. 3B, lane 13) pure.

Estimates of the amounts and proportions of protein, IgG and MSA in the major fractions obtained during purification of S5/7/7/6 IgG are tabulated in table 12 and are representative of the results obtained with other ascites sera. A number of technical difficulties, outlined in the footnotes to the table, make it extremely difficult to determine the quantitative composition of the more complex fractions reliably. There are three major sources of uncertainty. The differential response of proteins in the Bradford assay when total ascites protein is determined is unknown (cf. M.1.1.1). In addition differential staining of individual polypeptide bands by coomassie blue in SDS-PAGE electrophoretograms also occurs, (unpublished observations; RISAU 1983) but the extent of the differences for serum polypeptides has not been exactly determined. Lastly overloading of the gels, which is neccessary to see the light chains clearly, hinders reliable quantitative image analysis of photographic images of the stained gels. As a result there may be considerable errors (either over- or underestimates) of the protein levels in the AS fractions. Comparison of the estimate of total protein in the "cut" shown in table 12 with the sum of protein recovered from the chromatography of S5/7/7/6 on the S300 column (sum of pools A-F, lowermost separation in fig. 3A) reveals that only 72% of the material applied to the column is recovered. This low apparent recovery may be attributable in part to these problems, although failure to account for protein in fractions in which it was very dilute is also a source of error. The figures in the table must therefore be treated as only a very approximate guide. Nonetheless they make it clear that MSA is the major polypeptide component of ascites sera, making up 50% by weight of the total protein complement, whereas IgG initially accounts for at most 12% of the total. The table also shows that the whole procedure results in a 6-

Fraction	Volume (ml)	Total Protein[1] in fraction (mg)	IgG[1] (mg)	MSA (mg)	% Protein in Fraction IgG	MSA
Clarified Ascites	55	1530	180	770	12[2,3]	50[2]
60% AS Pellet	-	942[4]	180	370	19[5]	38[5]
25%-60% "cut"	14.6	807[4]	172	323	22[5]	40[5]
Sephacryl S300						
IgG Pool	63	157	126	8	80[2]	5[2]
MSA Pool	63	310	-	248	-	80[2]

Table 12 **Summary of amounts of protein recovered at different steps of the purification of S5/7/7/6 IgG from ascites serum using the "standard procedure". The Sephacryl S300 chromatography of this material is shown in fig. 3.**

Footnotes

[1] Protein is measured in units of "mg BSA equivalents" estimated using the Bradford method. To make the figures comparable in absolute terms another, underestimates resulting from the presence of IgG in fractions (see section M.1.1.1) have been corrected by using the formula: mg BSA equivalents (corrected) = mg BSA equivalents (measured) x corr., where corr.=0.54I+(1-I) and I is the decimal fraction (w/w) of the total protein represented by IgG.

[2] Based on quantitative image analysis of a negative photograph of the gel in fig. 3B. Correction factor for differential staining by coomassie blue assumed to be same as in Bradford assay.

[3] IgG has been estimated from the staining intensity of the light chain only, because non-IgG material coelectrophoreses with the IgG heavy chain. No attempt has been made to correct for any contribution to light chain staining by light chains from IgM and IgA (probably the source of the bands electrophoresing in the same position as light chain in lanes 5 and 6 of fig. 3B). This estimate is probably an overestimate: omitting the differential stain correction gives a value of 8% (120 mg) IgG.

[4] Values estimated by difference on the basis of the amount of protein found in the respective AS supernatant.

[5] Quantitative Image analysis not possible because light transmission of negative analysed not proportional to amount of protein applied to gel. (? saturation of dye binding to protein or of film response). Estimates based on 75% recovery of protein from column (probably results in overestimates).

to 7-fold purification of IgG which, because IgG is initially such an abundant protein, is near to the maximum achievable.

The figures which I obtain, 30 mg/ml total protein in clarified ascites and 3.3 mg/ml IgG are comparable with those obtained by FAHKRI (1970) who found 40 mg/ml total protein and up to 5 mg/ml IgG in the unclarified ascites sera of mice with well developed plasmocytoma ascites tumors. Given the inherent unreliability of the Bradford method for estimating absolute amounts of protein in complex mixtures (M.1.1.1) and assuming a small loss of protein (?10%?) upon clarification FAHKRI's results and mine can be considered to be in agreement.

The purpose of the AS precipitations, which is not immediately apparent from the results presented either in table 12, or from fig. 3B is twofold. Firstly, it serves to remove a number of undesirable components from the sera, and secondly it serves to concentrate the IgG. In the following two sections (R.3.3.1 and R.3.3.2) I outline the rationale for wishing to achieve these goals.

R.3.3.1 The advantages and problems of using ammonium sulphate fractionation to remove unwanted components from ascites sera

Many of the components in the initial ascites sera have low MW, such as the 10% protein which is dialysable (results not shown), colored materials and lipids. These components may not be separated by SDS-gel electrophoresis and in any case will not be visible using coomassie blue staining of electrophoretograms. They are nonetheless undesirable: the dialysable protein will react in the Bradford protein assay, the lipids cause undesirable surfactant effects, and the colored materials might interfere in subsequent colorimetric assays. Possible biological effects should also be considered if use of monoclonal antibodies in vivo is contemplated. Although I can produce no evidence for the removal of such components by

showing an SDS-PAGE electrophoretogram, nor indeed without resorting to a battery of more specific tests, it should not be forgotten that many of these components are likely to be soluble in 60% AS and would thus be be removed using the standard procedure.

Of the unwanted protein components in the serum about 50% of the absolute amount of MSA initially present is also removed by the first 60% AS precipitation step (table 12 compare MSA(mg) lines 1 and 2).

A further undesirable component which is reduced or removed by AS precipitation is a factor which interferes in radioimmunoassay for at least one Drosophila anti-nuclear antigen. Fig. 4 shows that specific activity of the antibody S5/7/7/6 increases disproportionately with purification and that the maximum amount of antibody which binds to antigen also appears to increase. Note that compared to the absolute amount of protein removed (table 12) the "cut" step, in which the initial 60% pellet is dissolved in 1/3 the initial ascites volume of 25% AS and any remaining pellet discarded, is particularly effective in removing this factor. The S5/7/7/6 antigen has since been found to be part of an RNA containing particle (RISAU 1983, RISAU et al., 1983), so a trivial explanation for the result shown in fig. 4 is that either an RNAase or a protease is being removed, but the observation does serve to underline the problems associated with interpreting data obtained with such complex mixtures as ascites sera.

Although the 25%-60% AS cut step was effective for fractionating the S5/7/7/6 antibody and most of the other monoclonals reported in this work (cf. table 13), subsequent experience has shown that this step can lead to large losses of IgG if used unwarily.

Both a monoclonal IgG3 antibody designated T61 (HENKE-FAHLE and BONHOEFFER 1983) and total mouse serum antibodies proved very poorly soluble in 25% AS. A control experiment in

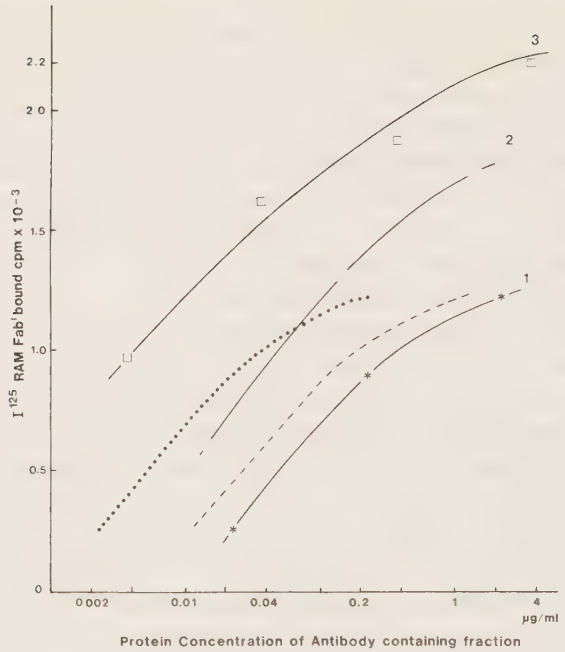

Fig 4 Increased activity of ammonium sulfate precipitated ascites sera in immunoradiometric assay (IRMA).

The various fractions were diluted in Iscoves medium containing 20% FCS and used in standard IRMA using sonicated Kc cells as antigen source. ^{125}I input was $1.2 .10^5$ cpm per well. The plotted points are means of duplicate values and have been corrected for blank (dilution medium alone with Kc cell nuclei). Curve 1, clarified ascites; curve 2, 60% AS pellet; curve 3, 28%-60% AS fraction. Dashed and dotted curves: simple extrapolation of curve 1 if antibody concentration in fraction were increased by a factor of 2 or 10 respectively.

which the 60% pellets of S5/7/7/6 and of T61 were redissolved in the same 25% AS solution showed that the S5/7/7/6 dissolved readily, as expected, whereas the T61 remained precipitated. Trivial effects could thus be ruled out. The poor solubility of the T61 antibody must have been an intrinsic property of the antibody itself. In the case of T61 the pH of the 25% AS solution (pH 7.1) may have contributed to its poor solubility

(see R.3.5.2). The T61 antibody has a pI > 8.0, but another antibody of the IgG2a subclass with a comparable pI (P11, pI 8.05) dissolves readily in 25% AS. Thus the poor solubility of the T61 antibody cannot be a direct result of its pI, but must be related either to its subclass or to its V-region properties.

Even more suprising is the observation that the bulk of mouse antibodies are poorly soluble in 25% AS. However in this case the effect seems to be mainly one of salt as the residue remaining in 25% was soluble in PBS. This means that the ready solubility of most of the anti-Drosophila antibodies is a rather uncommon characteristic of mouse antibodies. Perhaps it reflects some common functional characteristics.

In any case the variable behaviour of antibodies with respect to their solubility in 25% AS (either because of pH or because of AS) suggests that, in the interests of reproducibility in a "standard" method, the "cut" step should be omitted. Instead the initial 60% pellet should simply be redissolved, to make as concentrated a solution as possible, (adjusting the pH if neccessary) before it is chromatographed further.

R.3.3.2 Concentration of ascites serum by ammonium sulphate: a precondition for the efficient use of Sephacryl columns for IgG purification

Apart from removing undesirable components AS precipitation concentrates protein. There is an approximately 5-fold concentration of IgG following the final AS precipitation step. This feature of ammonium sulphate fractionation is important because it allows a Sephacryl column of feasible dimensions (< 1 metre length, 1.2 litre volume) to be used for the fractionation of up to about 50ml of hybridoma ascites sera (containing 100-200 mg antibody). Comparison of the $O.D._{280}$ elution profiles in Fig. 3A and of the polypeptide composition of the IgG pools obtained from these eluates shows (fig. 3B lanes 7 and 12 and fig. 8 for

P12/8/6) that the resolution of MSA and IgG is improved the smaller the volume that is applied to the column. In general, a volume not exceeding 1% of the total column volume should be applied. The 15ml used in separating S5/7/7/6 sets a barely acceptable upper limit to the volume that can be applied to the column to obtain adequate resolution. 50 ml is about the upper limit on the amount of ascites that can be purified at a single pass using this method with a column of these dimensions, because the concentration of total protein (approx 35mg/ml) and of IgG (approx. 10mg/ml) applied to the column is approaching the limit of solubility.

R.3.3.3 Summary of results of purifications of mouse monoclonal antibodies from ascites sera using the "standard" method

The yields of IgG purified from the ascites sera of a 17 hybridomas producing anti-Drosophila nuclear protein antibodies and of 1 plasmocytoma (P3-X63-Ag8), together with estimates of the original concentration of IgG in the ascites sera, are summarised in table 16.

All but two of the hybridomas gave rise to ascites with IgG concentrations in the range 1 mg/ml to 4.5 mg/ml. SDS-PAGE analysis of unprocessed ascites sera of the two hybridomas from which only very low amounts of antibody were recovered (Q18/5/4 and P46/6/7) showed that low concentrations of IgG in the starting material and not losses during purification were responsible for the low yields. Why these two hybridomas produce such low yields is unknown. "Loss" of antibody production may have been quantitative rather than qualitative. Specific antibody was detectable, at least from the Q18 ascites, by indirect immunofluoresence staining of Kc cell nuclei. This is the reason why the low antibody titre was not detected until IgG purification was attempted. The ability to produce IgG in large amounts may have been lost as a result of an instability similar to the one noted in R.1.6. It is also

Name of Hybridoma	Ig Class/ subclass	Volume Ascites (ml)	Recovery from S300 Column (mg)	Original Concentration in Ascites (mg/ml)
Ah6/5/9	IgM	2.0	n.a.	0.5
P11/3/6	IgG2a	90	180	2.0
Q18/5/4	IgG2a	42[1]	13	0.3 (0.15)[2]
S5/7/7/6	IgG1	55	157	3.3[3]
T7/3/3/4	"	17	59	3.4
P12/8/6	"	37	54	1.4
Z4/10/9	"	47	101	2.1
X4/8/6	"	48	72	1.5
P46/6/7	"	60[1]	10	0.17 (0.11)[2]
V4/6/9/1	"	39	81	2.0
W2/8/1/3	"	69	180	2.34
W12/10/6	"	55	248	4.5
T37/4/17/4	"	36	148	4.1
Aj1/2/2	"	20	50	2.3
T40/7/7	"	18	18	1.0
T50/7/11	"	18	36	2.0
U25/1/9/1	"	22	48	2.2
P3-X63-Ag8	"	83	129	1.6

[1] May include non-productive ascites.
[2] Total IgG (specific IgG) [3] see table 12

Table 13 **Purification of hybridoma antibodies from ascites sera by the "standard" method. Yields and estimated initial concentration of antibody in acscites sera.**

Unless otherwise indicated, estimates of original concentration are based on the assumption that recovery from the S300 column was 100% and that the IgG in the major peak was 90% pure.

possible (but unlikely, see below) that these monoclonal lines intrinsically produce very little antibody.

Some hybridomas are intrinsically poor IgG producers. The range of amounts produced by cells reaching a density of 10^6 cells/ml varies over a 100 fold range between 1 µg/ml and 100 µg/ml (FAZEKAS de ST.GROTH 1983; see also R.8). However, recloning of the Q18 hybridoma from earlier subclones gave

rise to clones able to produce as much as 40 µg/ml IgG in vitro. If these Q18 clones should continue to give such poor yields in ascites (there was no time to test this before completing the experimental work for this thesis) then poor production would have to reside in an interaction between this hybridoma and the ascitic environment. If this were the case there would little point in attempting to produce IgG from this hybridoma in ascites form.

In summary the production of IgG from 50 ml ascites sera by the "standard" method will gives yields of between 50 and 200 mg nearly pure IgG in 9 cases out of 10. Occasional low yields (10% frequency) can be attributed to poor IgG secretion by hybridomas and not to any deficiency in the purification method itself.

R.3.4 Highly purified antibodies

Although the standard method produces a fraction highly enriched in antibody, this degree of purification may still not be sufficient to allow antibody to be used for some of the purposes outlined in section R.3.1 (e.g. for production of Fab fragments R.3.4.5). For this reason this section describes methods for producing highly purified antibody.

R.3.4.1 IgM

Concanavalin A (con A) binds the IgMs of several species (LEON 1967 (man); NORDIN et al., 1969 (mouse); BOYLE and LANGONE 1978 (rabbit)) and affinity chromatography on con A-sepharose has been used to purify IgM obtained from human myeloma serum (KLEINE et al., 1979) and to isolate radiolabelled IgM from murine hybridoma supernatants (SCHULMAN and KÖHLER 1978). The IgM in the first of the reports cited had

already been substantially purified and IgM and IgG were the only labelled components found in culture supernatants under the conditions used by the other authors. Thus, while these reports show that it is possible to bind IgM to con A-sepharose and recover it, and that IgM can be separated from IgG, they do not indicate whether it is possible to use affinity chromatography on con A-sepharose to separate IgM from all other serum components. Con A is known to bind to a number of serum proteins (NAKAMURA et al., 1960; LEON 1967), but the affinity of proteins for con A is known to vary (ROGERS et al., 1974). It therefore appeared worthwhile to investigate whether it was possble to purify IgM by choosing the appropriate sugar concentrations to differentially release IgM bound to con A-sepharose columns.

Fig. 5 shows that it is. Panel a of fig. 5 shows that a fraction of the AS "cut" from the ascites serum of hybridoma Ah6/5/9 is retained on a con A-Sepharose column in the presence of 0.05 M α-methyl-mannoside but is eluted by a 0.5 M concentration of this ligand. The SDS-PAGE analysis shown in panel b provides evidence that this fraction contains IgM. A polypeptide electrophoresing in the position of the μ chain of IgM is prominent in the material applied to the column (fraction 0), but is depleted in the flow through (fraction 4) suggesting extensive binding of IgM. This conclusion is supported by semi-quantitative Ouchterlony analysis of the flow through fractions with rabbit anti-mouse IgM. This analysis shows that at most 10% of the IgM applied to the column can have been present in the flow through. The high molecular weight band which is present in the flow through in the same position as the μ chain is probably not IgM although the presence of polypeptides in the light chain region of the gel suggests that it might be some other kind of immunoglobulin (?IgA?). The pattern of the polypeptides present in fraction 17 in panel b shows that IgM is not eluted by 0.05 M α-methyl-mannoside although this concentration of sugar causes the release of a number of other ascites proteins from the column.

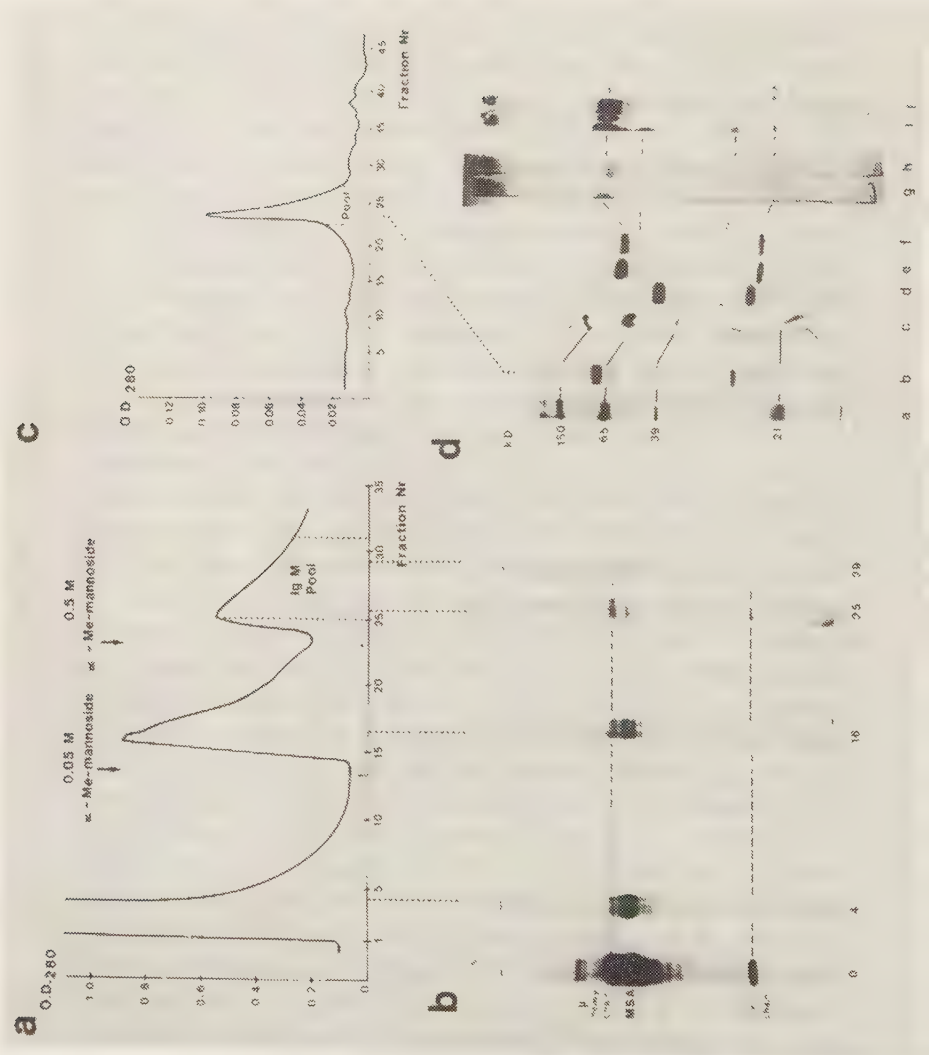

Fig. 5 (see next page for legend)

Fig. 5 **Purification of mouse IgM by affinity chromatography on con A sepharose.**

Panel a **O.D.$_{280}$ elution profile of a con A sepharose column.** 2 ml of a 25% to 60% AS "cut" (from 10 ml of Ah6/5/9 hybridoma ascites) was applied to a 3 ml con A sepharose column equilibrated in M buffer (M.2.3.3.3). Flow rate 1 ml/hr. 1 ml fractions were collected. At the eluate volumes shown by arrows above the profile the column was eluted with the indicated concentrations of α-methyl-mannoside in M buffer.

Panel b **SDS-PAGE of con A sepharose column fractions.** The indicated fractions from the profile shown in panel A were analysed without further concentration. Aliquot sizes: fractions 17,25,29 6 µl; fraction 4 and material applied to the column (=lane 0), 4 µl.

Panel c **O.D.$_{280}$ elution of 0.5 M con A pool on Sephacryl S300.** 1 ml of the pooled protein eluted from the con A sepharose column with 0.5 M α-methyl-mannoside was applied directly to a Sephacryl S300 column (2.6 cm diam. x 60 cm, 190 ml vol.), flow rate 8.75 ml/hr 2.5 ml fractions. The flow through spectrophotometer exhibits baseline drift at the high level of sensitivity used here.

Panel d **SDS-PAGE and immunoblot analysis of purified IgM.**
Lanes a,c: M.W. markers for lane b and for lanes d-f respectively (2 different gels). The M.W. shown in kilodaltons on the lefthand side of the panel correspond to the following marker proteins. 155 kD, RNA polymerase ß and ß' subunits; 65 kD, BSA; 39 kD, RNA polymerase α subunit; 21 kD trypsin inhibitor.
Lane b: peak fraction from S300 column, a 100 µl aliquot lyophilised and dissolved directly in SDS-PAGE sample application mixture.
Lane d: monoclonal IgG2a marker (P11/3/6), heavy chain 49 kD, light chain 28 kD
Lane e: IgM Ah6/5/9 con A 0.5M pool after AS precipitation and dialysis (30 µg applied to gel)
Lane f: IgM commercially purified material from plasmocytoma TEPC 183, used as reference material (30 µg applied to gel).
Lanes g-j: immunoblot of replicas to samples identical to lanes e and f developed with rabbit anti-mouse IgM, biotinyl goat anti-rabbit and avidin phosphatase.
Lanes g and h: phoshatase reaction after 30 min (g,left hand side TEPC183; h: Ah6/5/9)
Lanes i and j: phosphatase reaction after 16 hr (i as g, j as h).
MW's of 70 kD and 27 kD respectively have been assumed for the µ and light chains of IgM.
The horizontal black lines connect stained bands of the same MWs. The different dimensions of the blot and the gel are the result of different photographic enlargement.

A polypeptide chain with the same molecular weight as the µ chain is present in fraction 17, but since there are no corresponding light chain polypeptides in the fraction it cannot form part of an intact immunoglobulin. In contrast polypeptides with the molecular weight of both the µ chain and immunoglobulin light chains are almost the only species

present in the fractions eluted by 0.5 M α-methyl-mannoside.

Panels c and d present evidence which suggests that the protein present in the pooled fractions (25-29) containing material eluted by 0.5 M α-methyl-mannoside is indeed almost pure IgM. Firstly, when chromatographed on Sephacryl S300 the $O.D_{280}$ adsorbing material in this fraction chromatographs as a single nearly symmetrical peak at the expected elution position for IgM (panel c). IgG and serum albumin would elute after fraction 50 on this column. Secondly SDS-PAGE analysis of material from the pooled fractions concentrated either before or after S300 chromatography (panel d, lanes b and e) reveals two major bands with the molecular weights expected for the μ and light chains. The migration of these bands is identical to those in a reference sample of commercially avilable monoclonal IgM (panel d, lane f), and clearly differs from that of the component bands of a monoclonal IgG antibody (P11) which is shown for comparison (panel d, lane d). In addition the μ chain band of both the Ah6/5/9 preparation and the reference TEPC 183 preparation reacts strongly when they are immunoblotted with anti-IgM (panel D lanes g and h).

There is also a weakly staining band in the Ah6/5/9 preparation (panel d, lanes b and e) corresponding to a polypeptide with a molecular weight similar to that of MSA. However, since this polypeptide is not separated from the μ chain containing protein either by S300 chromatography, or by ammonium sulphate precipitation, it is unlikely to be MSA. Since it reacts weakly with anti-mouse IgM in the immunoblot analysis (panel D, lanes i and j), a more likely explanation would seem to be that this material is a "nicked" form of the μ chain itself (BÜSEN 1982). Indeed more of this material is present in the IgM standard than in the Ah6/5/9 preparation (panel d, lanes f and i).

The purified Ah6/5/9 antibody was still functional as judged by the pattern of indirect immunofluoresence to which it gave rise on Kc cells (FALKNER et al. 1982 and results not shown).

In summary, affinity chromatography of IgM containing ascites sera on con A sepharose under the conditions I have described is able to produce an almost pure preparation of mouse IgM. It remains to be seen whether other mouse IgMs will behave identically.

R.3.4.2 Protein A purification of mouse IgG subclasses

Since the initial report of Protein A binding to human antibodies (FORSGREN and SJÖQUIST 1966) this protein has been shown to bind to the antibodies of many different species (KRONVALL et al., 1970a) although its affinity for different subclasses of antibodies from the same species has been shown to vary considerably (for recent reviews see LANGONE 1982a,b). Conditions upon which binding is dependent, such as pH and ionic strength has also been investigated in detail a number of instances (e.g. for mouse, EY et al., 1978, SEPPÄLÄ et al., 1981; rat, ROUSSEAUX et al., 1981 and for a summary of results with other species see LANGONE 1982a).

Although Protein A Sepharose can be used to purify IgG directly from serum, I used it to remove contaminants from IgG which had first been extensively purified by the "standard" method. This procedure was adopted with the aim of preserving the column for repeated use. It was hoped that use of these more purified fractions would protect the protein A from proteolytic attack (MILLER and STONE 1978) and from the cumulative binding of other non-IgG serum components such as IgM (MACKENZIE et al., 1978; LANGONE 1982b).

The SDS-PAGE analyses presented in fig. 6 shows that IgG3 (lane 3) or IgG2a (lanes 4 and 5) can be obtained better than 99% free of other components from protein A sepharose, even when the material applied to the column contains numerous other components (lane 2). The separation procedure was based upon the conditions described by EY et al., (1978) or by SEPPÄLÄ et al.,(1981) for the elution of IgG2a or IgG3

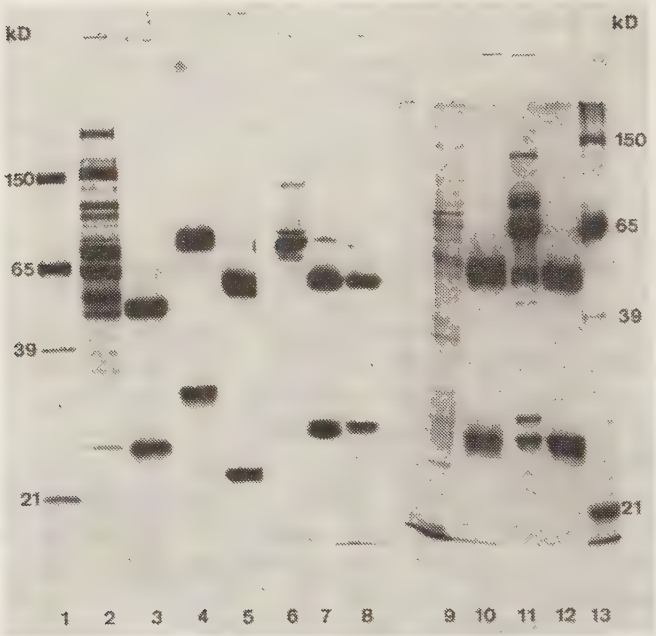

Fig. 6 **SDS-PAGE analysis of immunoglobulin purified on Protein A-sepharose.**

A 6.5 ml column was used for all the separations shown here. SDS-PAGE was in 10-17% linear gradient gels. See legend to fig. 5 (panel d) for details of marker proteins used in lanes 1 and 13. 5 separate electrophoretic separations are shown in lanes 1-3, 4,5, 6-8 and 9-13 respectively. Because of slightly different electrophoresis conditions (e.g. run time) and photographic enlargement factors, direct comparison of migration distances is only possible within and not between each of the 5 separations. Internal standards showed that the polypeptides giving rise to the upper and lower of the two most heavily stained bands in lanes 4,5 and 8 migrated in the correct position for mouse immunoglobulin heavy and light chain respectively.

The origin of the analysed protein fractions is as follows:

Lane 1) MW marker proteins

Lane 2) 20 µl of the flow through obtained when an IgG3 (T61) enriched fraction (25% AS pellet R.3.3.1) was applied to protein A sepharose in 100 mM citrate buffer pH 6.0.

Lane 3) 20 µl (approx. 15 µg protein) of a pH 4.5 eluate from the above column.

Lane 4) 30 µg P11/3/6 purified on protein A-sepharose

Lane 5) 30 µg Q18/5/4 purified on protein A-sepharose

Legend to Fig. 6 continued on next page

respectively. The antibodies thus prepared were not only freed from non-IgG contaminants but also from minor amounts of IgG of the IgG1 subclass (presumably normal constituents of mouse serum) which were also present in the the IgG prepared from ascites by the "standard" method (Ouchterlony tests, results not shown). pH 4.5 was used to elute both subclasses, thus any cross contamination of an IgG2a antibody by IgG3 or vice versa would not be removed under these conditions. This was not a practical problem since such contamination has been undetectable until now using Ouchterlony tests. However, if it had been a problem, differential elution of IgG2a (at pH 5.0) from IgG3 (at pH 4.5) could have been attempted if the IgG2a was produced by cells derived from mice with Igh "a" allotype (such as Balb/c mice). If the mice (?Tübingen mice?) have a non-"a" allotype then such a separatation is not possible because IgG2a and IgG3 would both be expected to elute at pH 4.5 (SEPPÄLÄ et al.,1981).

Because the eluate fractions were neutralised directly upon elution (see materials and methods) the affinity purified

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(Legend to fig. 6 continued from previous page)

Lanes 6) to 8) fractions from the protein A sepharose chromatography of the monoclonal IgG1 antibody V4/6/9/1 whose elution profile is shown in fig. 7 (see legend of fig 7 for details).
Lane 6) 20 µl pH 8.0 flow through fraction 5
Lane 7) 20 µl pH 8.0 flow through fraction 12
Lane 8) 20 µl pH 6.0 eluate (approx. 10 µg protein)
Lane 9) IgG1 antibody W2/8/1/3: 20 µl of flow through of an IgG pool (10 mg total protein in 20 ml) applied to protein A-sepharose in 100 mM Tris-citrate pH 8.2. All flow through fractions had a similar composition.
Lane 10) IgG1 antibody W2/8/1/3: 20 µl of a pH 6.5 eluate (approx. 15 µg protein). Total pH 6.5 pool approx. 8 mg protein.
Lane 11) IgG1 antibody S5/7/7/6: 20 µl of a flow through/retarded IgG1 fraction from a protein A sepharose column to which 24 ml of an IgG fraction containing 35 mg total protein had been applied in 100 mM Tris-citrate pH 8.2 (cf. lane 7)
Lane 12) IgG1 antibody S5/7/7/6: 20 µl of pH 5.7 eluate (approx. 30 µg protein). Total pH 5.7 pool approx. 9 mg protein.
Lane 13) MW marker proteins

antibody was only subjected to potentially denaturing acid conditions for a few minutes. This method is therefore very mild in comparison to previously described ones which used strong denaturing agents such as thiocyanate to elute bound IgG (MACKENZIE et al., 1978). An IRMA titration of P11/3/6 antibody applied to and eluted from such a column revealed no detectable loss of activity.

There are a number of reports that mouse IgG1 also interacts with protein A-sepharose (MITCHELL et al., 1977a,b, GODING 1978, MACKENZIE et al., 1978, EY et al., 1978, CHALON et al., 1979, SEPPÄLÄ et al., 1981) such that its appearance in the flow-through is retarded when it is applied to the column at pH 7.0 to 7.4 and that proportion of serum IgG also appears to bind under these conditions. DRESSER (1978) extended these observations and suggested that IgG1 is retained by protein A-Sepharose when it is applied to it in 10 mM Tris-Cl pH 8.0 and can be isolated free of other serum components and other subclass antibodies by elution at pH 6.0. Attempts were therefore made to purify IgG1 by following this procedure.

Unfortunately, DRESSER's procedure gives variable results and poor yields, although a small amount of very pure IgG1 usually was obtained in fractions eluted from protein A-sepharose by pH less than 6.5 (fig 6 lanes 8, 10 and 12). The cause of the problem seems to be that capacity of protein A sepharose for **retaining** IgG1 is very much less than for the other mouse antibody subclasses. This is illustrated in the $O.D._{280}$ elution profile of a fraction containing the IgG1 monoclonal antibody V4/6/9/1 which is presented in fig. 7 This shows that the $O.D._{280}$ of the flow through at first reaches a constant value and is depleted of IgG (fig. 6 lane 6). Thereafter the IgG1 antibody elutes in two peaks. The first leaves the column at pH 8.0 and has been retarded on the column by about 3 column volumes. Because of the volume

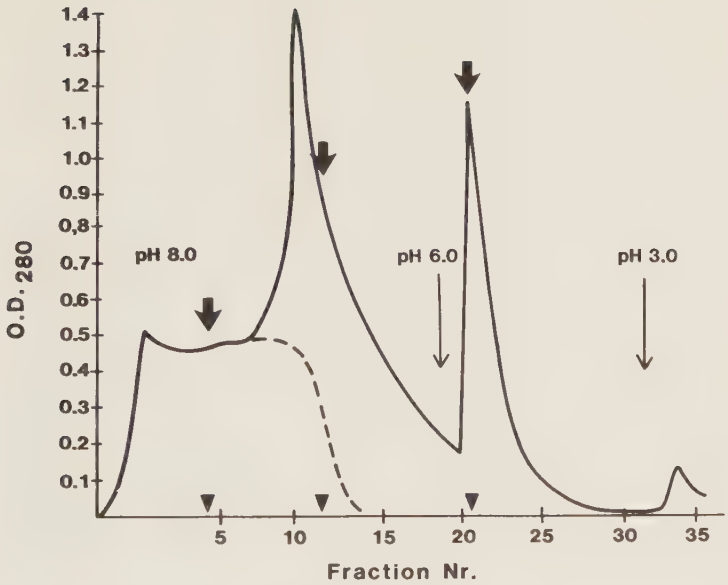

Fig. 7 **The binding capacity of Protein A Sepharose for mouse IgG1 antibodies is low.**

$O.D._{280}$ elution profile (tracing from flow-through spectrophotometer) of an S300 fraction of IgG from the hybridoma ascites V4/6/9/1 purified by the standard method, dialysed against 50 mM Tris-Cl pH 8.0 and applied to a 6.5 ml column of protein A sepharose equilibrated in the same buffer. At the points indicated by the thin vertical arrows the pH of the elution buffer was changed. Samples from the fractions indicated by the thick arrows were taken for analysis by SDS-PAGE (see fig. 6) Note that the flow rate was not uniform, although fractions of the same size were gathered. The predicted $O.D._{280}$ elution profile of the non-immunoglobulin proteins in the absence of IgG1 overloading is shown on the figure by the dashed curve. 26 mg protein was applied to the column. Estimated amounts of protein in the (theoretical) non-immunoglogulin fraction, in the first and second IgG1 peaks are 8 mg, 11 mg and 7 mg respectively.

applied to the column this material overlaps with the non-IgG flow through and remains contaminated (fig. 6 lane 7). The second is eluted at pH 6.0 and is >95% pure (fig. 6 lane 8). The amount of antibody in the pooled fractions containing the second peak was 7 mg. Similar amounts were found in the pooled material eluted below pH 6.5 using IgG fractions containing two other IgG1 antibodies S5/7/7/6 and W2/8/1/3 (see legend to fig. 6 for details). In two experiments in which less IgG1 was applied to the columns the first peak did not appear and the IgG1 flow through and the pH 6.0 eluate could be completely separated from one another (fig. 6 lanes 9 and 10).

Varying the conditions (pH between 8.0 and 8.5; ionic strength between 10 mM and 100 mM) did not appear to cause any substantial change in the behaviour of antibody. I have the impression that the capacity of the column may be marginally improved by using 100 mM Tris-citrate buffer for applying the antibody but I have not yet been able to test this idea rigorously. I also suspect that the concentration at which the IgG1 is applied may be important.

A column capacity of 1 mg/ml (packed column volume) is only 5% of the column capacity (20 mg/ml) claimed by the manufacturer for human IgG. It was also clearly less than the capacity of the column for the IgG2 and IgG3 antibodies. Using IgG from total mouse serum I was able to bind IgG2 and IgG3 at at 4 mg/ml column volume. However, since I have not attempted to use higher loadings, it is possible that the maximum capacity is still greater.

Close examination of the reports by EY et al., (1978), MACKENZIE et al., (1978) and SEPPÄLÄ et al., (1981) shows that these authors all used very low column loadings to examine the binding behaviour of the different subclasses of serum IgG to their columns, so they would not have noticed the problems encountered with IgG1. Their results do however show that there is a wide range of variation in the tenacity with which

IgG1 antibodies are able to remain bound to protein A-sepharose. Some antibody is unable to bind at pH 7.0 whereas a small but measurable amount is still retained below pH 6.0. Both EY et al., (1978) and GODING (1978) have commented on this behaviour and suggested that there may be subdivisions of this subclass which behave differently.

It is therefore possible that the problem of finding a single set of conditions for the efficient purification of IgG1 antibodies on protein A-sepharose will prove intractable. Both the lower capacity of protein A sepharose for the IgG1 subclass and its variable retention on this medium differentiate it from the other subclasses, suggesting that its members may bind to protein A by a different mechanism. If mouse IgG1 antibodies are able to bind via their $(Fab')_2$ moieties rather than their Fc portion (INGANÄS et al., 1980) then almost every antibody might behave slightly differently. It should be possible to test this proposition by testing the binding behaviour of a series of IgG1 monoclonals under identical conditions.

In summary, it appears that while there are few problems in using protein A-sepharose to purify IgG2 and IgG3 antibodies in practical amounts. IgG1 can be purified using this medium but it is neccessary to restrict the amount of IgG1 antibody applied to the column to avoid overloading and poor separation. If attempting to purify a previously untested IgG1 antibody, it may also be advisable to restrict the **volume** of antibody containing material applied to a protein A-sepharose column to about one column volume in case of weak binding.

R.3.4.3 Chromatofocusing of IgG

In the previous section (R.3.4.2), it was noted that capacity of Protein A sepharose for binding IgG1 was low and may vary from antibody to antibody. An attempt was therefore

made to find an alternative purification method for IgG1 antibodies. The method of "Chromatofocusing" (PHARMACIA) which is able to separate proteins according to their isoelectric points (IEPs) was evaluated for its suitability. If this method were to work according to the manufacturers claims it would offer the advantage of being able to resolve a mixture of antibodies if their IEPs were different. Since some hybridomas do secrete mixtures of antibodies (see section R.3.5.2) this is of practical importance.

The principle of this method of separation is as follows. Proteins are bound to a weak ion exchange medium within a column in a high pH buffer (start buffer). A mixture of polyelectrolytes (Polybuffer - trade mark), specially developed for use in conjunction with the ion exchange matrix and at lower pH (elution buffer), is then allowed to flow through the column such that a pH gradient forms within it. The gradient moves along the length of the column until the whole column attains the lower pH. As bound proteins are subjected to the pH of their IEP their net charge is by definition nil, their binding to the charged groups on the ion-exchanger is at its weakest and they detach from the matrix and eventually leave the column in a small volume of elution buffer with a pH at or near their IEP. By varying the pHs of the start buffer and the elution buffer, the species of proteins bound to the column initially and the steepness of the pH gradient can be manipulated. The resolution between proteins with different IEPs can thereby customised to the particular separation required.

Figure 8 shows an example of chromatofocusing using the IgG fraction isolated from ascites of the IgG1 hybridoma P12/8/6 by the "standard" method (cf. fig. 3). SDS-PAGE analysis of the material applied to the chromatofocusing column (lane A on the right hand side of the figure) shows that although this fraction mainly comprises the heavy and light chains of IgG it also contains mouse serum albumin (MSA), another high molecular weight polypeptide and a number

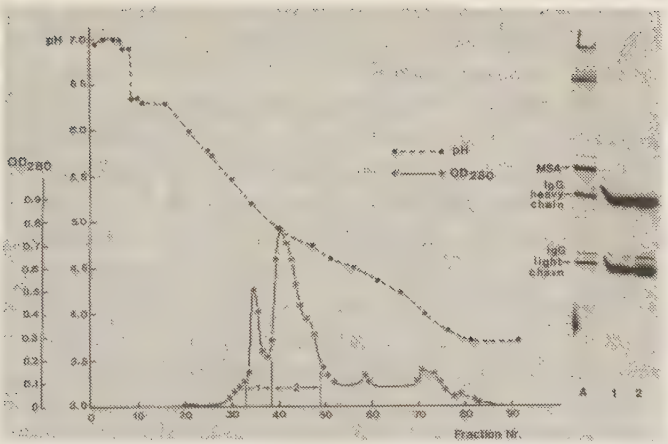

Fig. 8 **Chromatofocusing of the IgG1 monoclonal antibody P12/8/6.**
An IgG enriched fraction was prepared from ascites serum by the "standard" method (cf. fig. 3). 20 ml of this fraction in 1/10 diluted PBS was applied to a 40 ml column of PBE 94 (Pharmacia) equilibrated with 25 mM Imidazole buffer pH 7.4. Elution was carried out using a polybuffer mixture adjusted to pH 4.0. Other conditions as detailed in materials and methods, except that the $O.D_{280}$ of individual fractions was measured by hand rather than using a flow through spectrophotometer.
Left hand side of figure: **$O.D._{280}$ and pH elution profiles**
Right hand side of figure: **SDS-PAGE analysis**
Lane A, material applied to column (10 µg total protein).
Lanes 1 and 2, material eluted from the column, corresponding to pools 1 and 2 indicated in the $O.D._{280}$ profile, (50 µg protein/lane) the chromatofocusing column. Pools 1 and 2 were electrophoresed after ammonium sulphate precipitation and dialysis against 1/10 diluted PBS. Electrophoresis of the applied material was in a 12.5% gel and of the eluted material in a 7%-17% linear gradient gel (courtesy of W. RISAU). Heavy and light chains of IgG were identified by running a standard preparation of IgG in the same gel (not shown). The coincidence of the position heavy and light chains in each of the two gels is fortuitous.

of polypeptides with MWs intermediate between the heavy and light chains. The $O.D._{280}$ elution profile of this material shows that it is resolved into at least two peaks of material absorbing strongly at this wavelength. SDS-PAGE analysis of the pooled fractions corresponding to these peaks (lanes 1 and

2 on the right hand side of the figure) show that the protein in each of these peaks does not differ in polypeptide composition but that MSA and and two other contaminating polypeptides have been removed by chromatofocusing. The IgG in both pools was active in IRMA and probably repesents isoelectric point variants of the P12 antibody. The minor polypeptide bands which co-chromatofocus with IgG make up at most 10% of the protein in each pool (see also the non-overloaded SDS-PAGE analysis in fig. 10) and have not been characterised further. However, when the (native) antibody is analysed by isoelectric focussing no additional bands are observed which suggests that the minor polypeptides seen in SDS-PAGE might be still be the part of the native antibody. They might represent "nicked" heavy chains analogous to the "nicked" polypeptide chains which have also been observed in purified preparations of functional enzyme molecules (BÜSEN 1982).

The recovery of protein in the two IgG containing peaks was approximately 80% (table 14) of the material applied to the column. This is approximately the IgG content of the material applied to the chromatofocusing column and shows that chromatofocusing can be an efficient method for producing highly purified antibody.

Details of the chromatofocusing conditions for the isolation of this and four other monoclonal antibodies are given in Materials and Methods (M.2.3.3.5) and the pH and yield with which they elute from the column is listed in table 14. The pH values of the eluates were very carefully measured. In one case two different pH meters were used and agreed with one another to within ± 0.1 pH units. The variation in yield between different antibodies probably results in part from differences in the degree of contamination of the IgG enriched fractions applied to the chromatofocusing column. The Sephacryl S300 IgG fractions of the antibodies Z4/10/9 and X4/8/6 were not so well resolved as those of the P12/8/6 and T7/3/3/4 preparations (cf. R.3.3). However some IgG may have been lost on the column. The Z4/10/9

Hybridoma	pI by IEF	pH at which antibody eluted	Protein applied (mg)	Antibody recovered (mg)	Yield (%)
P12/8/6	6.5-6.8	4.9 (5.2)[+]	36	28[*]	77
Z4/10/9	6.3-6.5	4.5	98[++]	40	41[++]
T7/3/3/4	5.5-5.8	5.0	60	52	81
X4/8/6	6.2-6.3	5.5	30	15	50
S5/7/7/6	5.9-6.8[1]	4.5-4.7[2]	7.8	6.6	84

[+] minor peak [*] sum of major and minor peaks
[1] Group of 3 isoelectric point variants [2] Small column
[++] May be incorrect, light scattering error because of incipient precipitation.

Table 14 **Elution pH and yields of monoclonal antibodies purified by chromatofocusing.**
Chromatography conditions are described in full in section M.2.3.3.5.

antibody preparation was poorly soluble in low ionic strength buffers (see R.3.5.3). In addition some IgG (e.g. non-hybridoma mouse IgG) may have been lost on the column.

Although individual antibodies are not eluted at their isoelectric points (pIs) as determined by isoelectric focusing, (table 14 and R.3.5.1), Chromatofocusing is capable of separating proteins differing in pI by only 0.1 or 0.2 pH units. This is illustrated in fig. 9 which shows the $O.D._{280}$ elution profile of 3 isoelectric point variants of IgG1 obtained from the ascites of the hybridoma S5/7/7/6 together with an IEF analysis of fractions corresponding to different positions within the "peak" thus obtained. The relative amount of each of the three IEP "families" (see sections R.3.5.1 and R.3.5.2) in each of the protein containing fractions clearly changes systematically as the pH of the eluate falls from pH 4.7 to pH 4.5. This demonstrates the potential of chromatofocusing for separating mixtures of antibodies of the same subclass. The antibody "families" were not completely resolved under the conditions used here, but

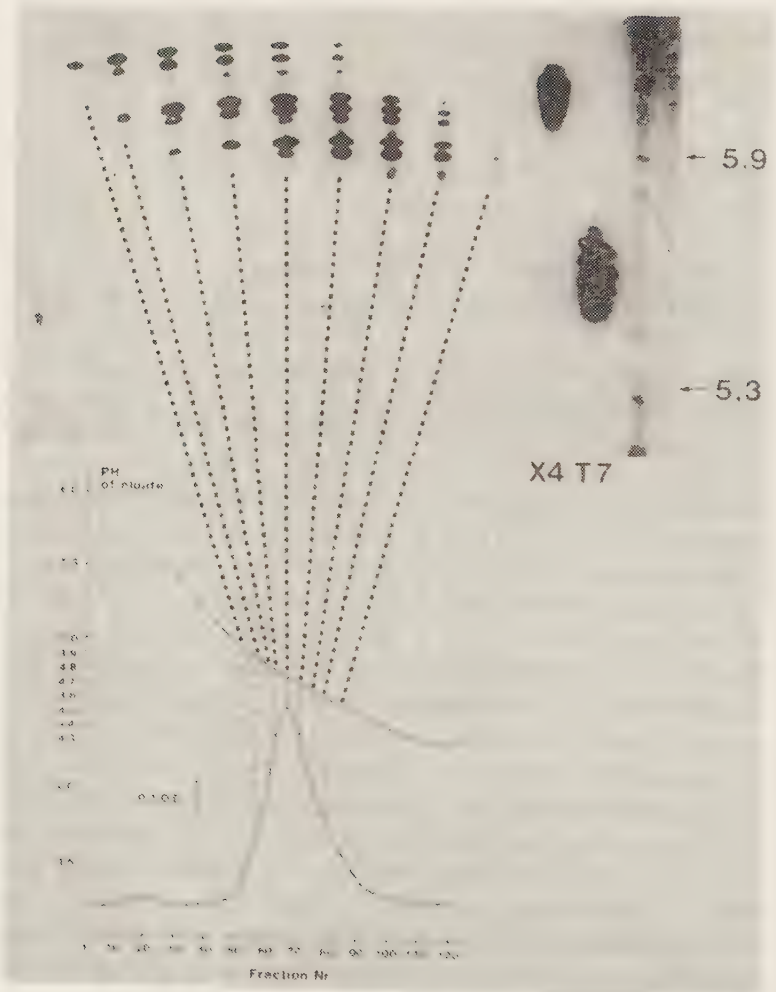

Fig. 9 (see next page for legend)

making the gradient shallower and possibly increasing the length of the column would probably achieve this.

Unfortunately, apart from this potential ability to resolve proteins with very similar charge properties, which might not be very much greater than that of a conventional ion exchange column (this would have to be tested in a direct comparison), chromatofocusing has two undesirable characteristics which make its use for separating antibodies difficult to recommend.

The first is that the lack of correspondence between the isoelectric point determined by isoelectric focusing and the elution pH (table 14) means that the conditions for elution cannot be predicted in advance and that the optimal elution conditions for each antibody would have to be determined by time consuming preliminary experimentation.

The second disadvantageous feature of chromatofocusing is the problem of removing polybuffer fully from the isolated IgG. Polybuffer is a mixture of synthetic polypeptides and according to manufacturers instructions they can be effectively removed by AS precipitation of the antibody, discarding the polybuffer in the supernatant. At first sight

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Fig. 9 **Separation of isoelectric point variants of mouse IgG1 variants by chromatofocusing.**

The isoelectric focusing pattern of individual fractions from a chromatofocusing column has been superimposed on the $O.D._{280}$ elution profile of the column. Correspondence between individual fractions and focusing patterns is shown by the dotted lines. Also present in the focusing gel are samples of antibodies X4/8/6 (pI 6.2-6.3) and T7/3/3/4 (pI 5.5-5.8) and a marker mixture (for details of composition see legend to fig. 15) from which only the positions of the pI 5.3 and 5.9 markers is shown.

The chromatofocusing was carried out in a 10 ml column, using the conditions indicated in the text (tables 6 and 14), to which was applied 2 ml of a 3.9 mg/ml of an S5/7/7/6 antibody preparation previously purified by low resolution preparative isoelectric focusing in a thin layer of Sephadex (courtesy of W. RISAU). 0.5 ml fractions were collected and 10 µl aliquots of each applied directly to the focusing gel. The focusing gel contained a 3:1 mixture of 5-7 and 2-11 servalytes and was focused until the pI 9.3 coloured marker (cytochrome c) had migrated out of the gel.

IgG treated in this way seemed adequately pure (fig. 10, lanes 1-4, 6 and 7). No stained material could be seen in the low M.W. region of SDS Polyacrylamide gel electrophoretograms in which free polybuffer can be detected (fig. 10, the very intense staining at the bottom of the gel in lane 10 is caused by free polybuffer). However, some anomalous properties of the purified antibodies subsequently suggested that a fraction of polybuffer was not removed by this treatment and bound very strongly to IgG under low ionic strength conditions.

The anomalous properties which provided the evidence for this binding were as follows:

1) bands on SDS-PAGE elecrophoretograms were stained more intensely with coomassie blue than would be expected on the basis of the amount of protein (determined by $O.D._{280}$) applied to the gel (fig. 10 lanes 1-4, 6 and 7) or when compared with the staining of identical amounts ($O.D._{280}$) of IgG which had not been chromatofocused (fig. 10 lanes 5 and 8);

2) the $O.D._{230}/O.D._{280}$ ratios of the chromatofocused antibodies was very variable (between 5.5 and 6.5) and exceeded the value obtained with non-chromatofocused IgG (5.2): polybuffer absorbs light at $O.D._{230}$ but not at $O.D._{280}$;

3) estimates of the amount of protein in chromatofocused antibodies using the Bradford method were greater than those obtained by measuring UV absorbtion at 280 nm. Polybuffer alone causes a powerful reaction the Bradford assay.

A reconstruction experiment in which SDS-PAGE was performed on non-chromatofocused IgG which had been deliberately mixed with polybuffer showed that the staining intensity of the electrophoresed polypeptides was indeed increased by polybuffer (Fig. 10 compare lanes 9 or 10 with lane 12). This means that polybuffer must be bound to both the heavy and light chains of IgG despite the presence of excess SDS and despite boiling of the IgG in SDS and mercaptoethanol to separate the chains.

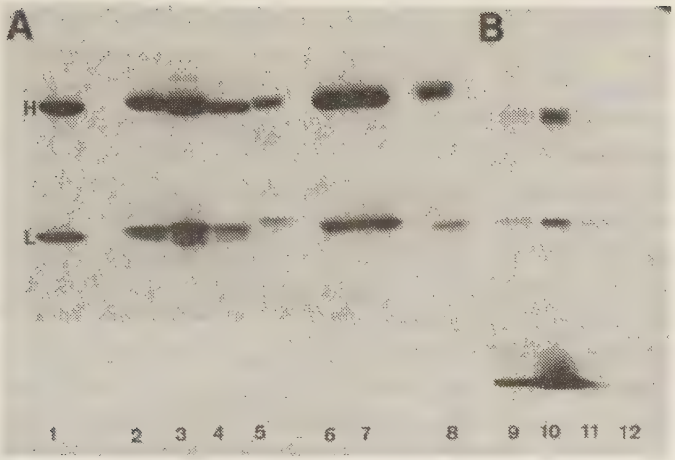

Fig. 10 **Polybuffer complexed to IgG causes artefactual staining by coomassie blue.**

Panel A **Chromatofocused IgG stains more intensely with coomassie blue than conventionally purified IgG.**

Equal amounts of purified antibodies (5 µg/lane) as measured by light absorbtion at 280 nm were subjected to SDS-PAGE on a 12.5% polyacrylamide gel. The IgG in lanes 1-4 (hybridoma antibodies P12/8/6, T7/3/3/4, X4/8/6, Z4/10/9 respectively) and in lanes 6 and 7 (plasmocytoma antibody MOPC21 from the cell line P3-X63-Ag8 - 2 lanes) had been purified by chromatofocusing. The IgG in lanes 5 and 8 (hybridoma P11/6/3 and commercially obtained mouse serum IgG) had been conventionally purified. H=IgG heavy chain L=IgG light chain.

Panel B **Mixing conventionally purified IgG with polybuffer increases the staining intensity by coomassie blue.**

4 identical samples of a preparation of P11/6/3 with degraded heavy chains (R.3.4.5, hence the smeared upper bands), each containing 2.5 µg in 20 µl distilled water were mixed with polybuffer as described below, 10 µl SDS-PAGE sample application mix was added and the samples reduced and denatured for electrophoresis in the usual way. Electrophoresis was in 12.5% polyacrylamide gel. Polybuffer (7 µl of a 1/10 dilution in distilled water) was added as follows: Lane 9) 1:1 mix of polybuffer 96 and 74
Lane 10) polybuffer 96
Lane 11) polybuffer 74
Lane 12) none (distilled water control)

The staining of the untreated control appears particularly weak because the gel has not been adequately destained.

Finally, ammonium sulphate precipitation of chromatofocused IgG followed by gel filtration on Sephacryl S200 in the presence of 2M NaCl gave rise to two peaks of $O.D._{230}$ absorbance in the eluate (not shown). The first one was from fractions eluting near to the exclusion volume of the column which contained the IgG. The second, smaller one, arose from fractions where lower molecular weight material would be expected to elute and which was presumably from free polybuffer. The IgG contained in the fractions giving rise to the first $O.D._{230}$ absorbance peak no longer exhited any of the anomalous properties listed above.

There is therefore little room for doubt that polybuffer and IgG can form very stable complexes. Interestingly the adsorbed polybuffer did not seem to alter the properties of the antibodies in IEF gels, perhaps because the large amount of polyelectrolytes in these gels is able to compete with bound polybuffer. With hindsight, the explanation for complex is almost certainly trivial: presumably sufficient polybuffer is trapped in the initial AS pellet to cause later complex formation and the problem could be avoided by repeatedly precipitating and redissolving the antibody. However, this is not the point of these observations, but rather the subtle nature of the complex which made it hard to detect in the first place.

Because of the subtle nature of the contamination most of the IRMA assays made in the HnRNP investigations of RISAU (1983) and RISAU et al., (1983) were made using polybuffer contaminated antibodies before the contamination was detected. Control experiments showed that, luckily, the presence of polybuffer in the antibody preparations did not affect the conclusions which were drawn from these studies. Indirect immunofluoresence staining of cells and chromosomes also appeared to be unaffected by this contaminant. Nonetheless one might expect that this contaminant would cause problems under other conditions. For instance, if antibody were microinjected into cells, or if derivatisation of antibodies was attempted

with a reagent which could also react with functional groups on polybuffer.

In summary, although chromatofocusing can provide an alternative method for the purification of mouse IgG antibodies and provides a means for separating mixtures of antibodies belonging to the same subclass it cannot be recommended for general use. The anomalous elution properties of IgG1 on this media, the danger of replacing detectable contaminants by undetectable ones and the extra steps involved in separating IgG from strongly bound polybuffer mean that considerable effort is required to obtain a separation using this medium. In terms of convenience, purification of IgG1 molecules on protein A columns is now preferred, despite the low capacity of protein A for this subclass of antibody.

R.3.5 Characteristics of highly purified mouse monoclonal antibodies

R.3.5.1 Isoelectric points and microheterogeneity

The purification procedures and SDS-PAGE analyses described in the previous sections allow the preparation of a pure immunoglobulin fraction which can be shown to be homogeneous with respect to the molecular weight of its constituent polypeptides. This does not necessarily mean that these fractions will be homogeneous at the molecular level i.e. that the fraction comprises an antibody with a single amino acid sequence. It would hardly be practical to control immunological purity by sequencing each preparation, but it is possible to control whether IgG preparations contain mixtures of antibodies whose sequence differences are great enough to give differences in net charge. Immunoglobulin charge variants can conveniently be characterised by isolectric focusing in thin layer polyacrylamide gels (AWDEH et al., 1968). Native monoclonal antibodies characterised in this way are known to

exhibit microheterogeneity giving rise to a set of 3 or more protein bands clustered with a 0.2-0.3 pH unit region (AWDEH et al., 1970). Microheterogeneity arises naturally because of variable deamidation of glutamine and asparagine residues following secretion (AWDEH et al., 1970; WILLIAMSON 1978) and possibly also because of variable sialylation of the carbohydrate part of the molecule (MELCHERS et al., 1966; WILLIAMSON et al., 1978). However the cluster of bands corresponding to each monoclonal antibody is spread over a reproducible range of isoelectric points still allowing each antibody to be characterised by the "spectrum" with which it focuses.

Apart from allowing the molecular purity of an IgG preparation to be controlled, there are at least two other advantages which accrue from knowing the pI of a monoclonal antibody. Firstly, the pI provides a criterion for identifying a monoclonal antibody, other than the antigen against which it is directed. This might be of use in deciding whether different subclones from an initial clone are directed against different antigens, if those antigens only exhibit small differences in molecular weight. It also provides an additional safeguard for the control and avoidance of contamination between hybridoma lines. Secondly, it should be possible to explain or predict variation between antibodies arising as a result of charge-charge interactions (e.g. unspecific binding, see R.7.3.1.2.3).

Fig. 11 shows how 6 highly purified monoclonal IgG preparations (T7/3/3/4, S5/7/7/6, X4/8/6, P11/6/3, P12/8/6 and Z4/10/9) and the IgG fraction from an S300 column prepared (P3) migrate under IEF conditions. The T7/3/3/4, Z4/10/9 and and P3 preparations each give rise to a set of 3-4 bands and each set is focused over a at different range of positions. The P12/8/6 antibody preparation from pool 2 of the chromatofocusing column (R.3.4.3) gives rise to more bands (6) but they all appear to be related. The slight shoulder in the elution profile shown in Fig. 8 may be a reflection of this.

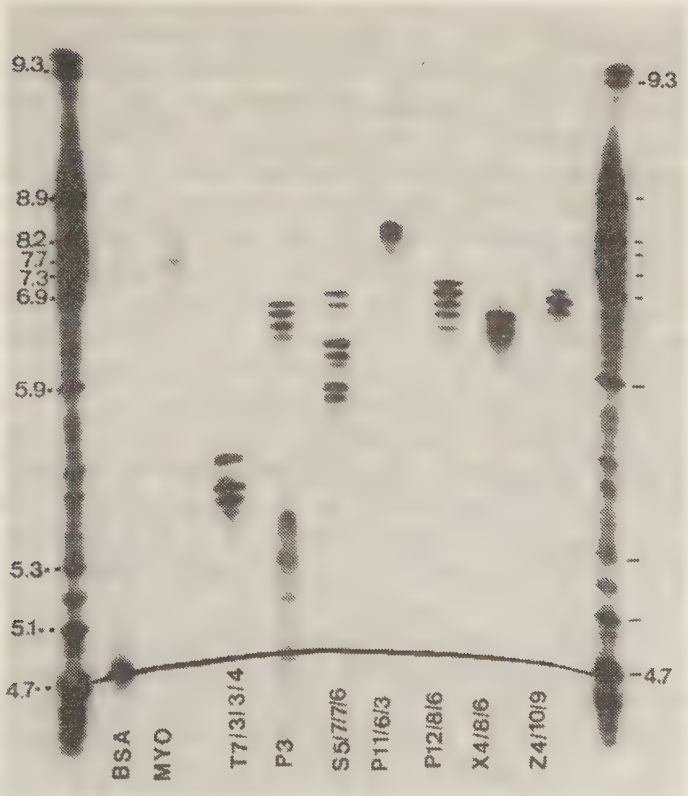

Fig. 11 **Isoelectric focusing of purified monoclonal antibodies.**

Isoelectric focusing was carried out in a 4.6% 1 mm thick polyacrylamide gel containing 4M Urea. The gel contained a mixture of pH 2-11, pH 4-6 and pH 5-7 "Servalytes" mixed in the ratio 2:1:1. For other details see M.1.4.2. after 15 min prefocusing at 300V the gel was run at 500V overnight and for 1 hr at 800V. The gel was cooled to 4 °C. After running 0.5 cm strips were cut from either side of the gel for pH determinations and the gel then fixed and stained with coomassie blue. 5 µl of a mixture of marker proteins (composition see legend to fig. 15, pI 9.3 marker is cytochrome c) has been run in the two outermost lanes. Pure samples of BSA and horse myoglobin were run in the lanes labelled BSA and MYO (the middle band in the MYO lane corresponds to the component referred to in the literature as "main band"). The designations of the monoclonal antibodies applied to the gel are indicated on the figure on the lanes in which they were run. Approximately 20 µg of each antibody was applied to the gel. The thick hand drawn black line at the cathodic end of the gel indicates the estimated position of the isoelectric front.

KÖHLER et al., (1976) show an IEF gel in which a similar number of bands have been obtained from the P3 monoclonal antibody and remark that the large number of bands is "due to ageing" of the antibody preparation. The P11/6/3 and X4/8/6 preparations are smeared. The X4 preparation did not migrate in this way in other gels, but gave a typical 3 band pattern. The P11 preparation may have behaved this way because it had not reached equilibrium and was not yet properly focused (see below). Lastly the S5/7/7/6 preparation shows 3 distinct groups of 3 to 4 stained protein bands. This almost certainly indicates that the S5/7/7/6 IgG fraction was heterogeneous and contained 3 different molecular species of IgG (see next section R.3.5.2 for further details).

Thus, apart from the S5/7/7/6 preparation and apart from the expected microheterogeneity, the monoclonal antibodies prepared by the methods described in the previous sections appear to be not only pure but also molecularly homogeneous.

Using 1 mm thick flat bed gels, the cooling equipment and 1000V max. power supply initially available (1 mm system), it proved difficult to determine the absolute values of the isoelectric points of the purified monoclonal antibodies with certainty. The reasons for this were technical.

Firstly, as can be seen from the tilted bands in the marker lanes of fig. 11 and more clearly in the parallel marker lanes of the focusing gel shown in fig. 15, the distance migrated by a protein from from the cathodic end of the gel was non-uniform across the breadth of the gel, giving rise to a bowed migration front. The estimated correction for this non-uniform migration is shown in the fig. 11 by the hand drawn black line.

Secondly, it was not possible to be sure whether the immunoglobulins, especially the more basic ones, had been focused to equilibrium, i.e. until they reached the position in the pH gradient in the gel where they carried no net charge. As fig. 12 shows the pH gradient within the gel only

reached pH 8.0, despite the inclusion of basic ampholytes in the gel. Although basic proteins migrate towards the anode and become focused i.e. the bands containing protein become narrower, the focusing is not isoelectric. The position to which they migrate clearly departs from that which they would be expected to reach at equilibrium, under which condition most of them would have migrated out of the gel. Most of the immunoglobulins migrate to positions at which migration of the markers begins to deviate strongly from the expected equilibrium position. Individual immunoglobulins might have different migration rates (e.g because of different charge to mass ratios). It is therefore questionable whether the migration of each would deviate equally strongly from the the equilibrium position. For this reason it is doubtful whether position relative to the isoelectric marker proteins can be used to **accurately** estimate the absolute isoelectric point of a monoclonal antibody. Furthermore, the position reached by the marker proteins was dependent upon the position at which they were applied to the gel, i.e. the colored marker myoglobin applied at either the anodic or cathodic end of the gel reached a different position. This would probably not affect the determination of the pI of an unknown protein using a calibration of migration distance against marker isoelectric points if all proteins were applied to the gel in the same position, but it does underline the purely relative nature of this calibration.

Poor formation of a pH gradient at the basic end of a focusing gel is often attributed to the presence of urea (PETERS and COMINGS 1980, UI 1971) but in control gels, in which glycerol or sucrose was substituted for urea, no difference in the development of the gradient was noted. Varying the ampholyte composition by increasing the proportion of basic ampholytes also caused little improvement. CO_2 absorbed into the more basic region of the gel is another factor which is suspected of depressing pH gradients (WILLIAMSON 1978), but I was not able to test whether this was

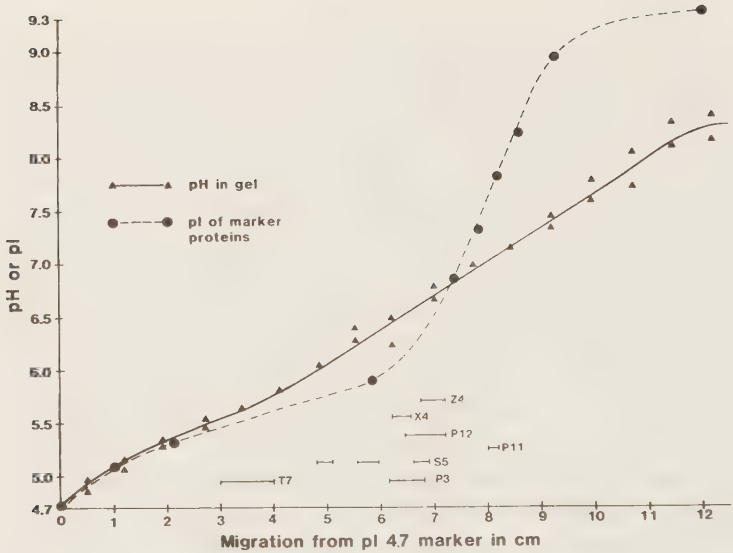

Fig. 12 **The electrophoretic focusing of basic proteins in a 1 mm gel system is not isoelectric.**

Measurements were made on the gel shown in fig. 11. The solid line shows the pH in the gel measured by eluting small segments cut from either side of the gel (M.1.4.2) into distilled water. The solid triangles show the measured data points. Where only a single triangle is shown the pH in the segments on both sides of the gel was identical. The dashed line shows the gradient followed by the focused marker proteins. Migration distances have been measured with respect to the anodic edge of the hand drawn line on fig. 11. The position at which each of the monoclonal antibodies migrated is indicated in the lower half of the figure.

the cause of the gradients I obtained. Changing the running conditions (voltage, power, run time) caused little improvement. Longer run times at 900 or 1000 V caused the curve of marker protein pI against migration distance to become shallower, but the pH gradient also became shallower so the two gradients never became superimposable. The inability to bring the two gradients into correspondence may have been the result of using a power supply unable to deliver more than 1000 V, because after a time the movement of the coloured marker proteins at this voltage was almost imperceptible.

The quantitative effect of these shortcomings is probably small. Shortly before completing this work it was possible to make a single experiment using a very thin layer gel (0.1 mm), in which most of the problems associated with the 1 mm gels were overcome (i.e the migration front was straight and proteins focused to the same position irrespective of the of the gel to which they had been applied). The result indicated that for proteins with a pI of less than 7.0 there was a systematic discrepancy between the two systems of about 0.2 pH units (1 mm gels giving lower pIs) when the pIs of the IgGs were estimated by using the migration of the marker proteins to calibrate the gel. The estimate of pI for P11/6/3 showed a still greater discrepancy (0.5 pH units). Most of the IgGs examined have pIs less than 7.0. Thus, provided that the migration of pI markers rather than the pH of the gel is used to calibrate pI, the errors introduced are sufficiently small to be able to use estimates from the 1 mm system at least as acceptable approximations of the absolute pI of IgGs.

Taking account of the above limitations it is thus still possible to obtain estimates of the pIs of the IgGs used in this study. These are shown in table 15 (next page) and are based on several focusing determinations. It is interesting to note that the two most basic antibodies (P11/6/3 and T61) are also those which exhibit the highest unspecific binding to the acidic protein BSA (pI 4.7) in ELISA (R.7.3.1.2.3).

R.3.5.2 Hybridomas producing three kinds of antibody

It is quite clear from the isoelectric focusing patterns shown in fig. 11 and from the chromatofocusing shown in fig. 9 that the S5/7/7/6 antibody preparation consists of three subpopulations of antibodies carrying different charge. The most likely explanation for this observation is that the S5/7/7/6 hybridoma secretes two different kinds of light

Hybridoma	Isoelectric Point (Range)
P11/6/3	8.3
T61	8.3
U25/1/9/1	7.8
Aj1/1/2	6.9
Q18/5/4	6.7-6.9
P12/8/6	6.2-6.8
S5/7/7/6(top)	6.5-6.8
V4/6/9/1	6.1-6.7
Z4/10/9	6.3-6.5
S5/7/7/6(mid)	6.1-6.3
P3-X63-Ag8[1]	6.2
X4/8/6	6.2
W12/10/6	6.2
P46/6/7	6.1
T50/7/11	6.0
S5/7/7/6(bottom)	5.9-6.0
T7/3/3/4	5.5-5.8

[1] Cell line derived from MOPC 21 Plasmocytoma

Table 15 **Isoelectric points of purified monoclonal antibodies.** These values are best estimates based on the results of a number of different isoelectric focusing gels. Their relative order is correct and the absolute values have been adjusted to correspond with those obtained by focusing in very thin gels.

chain: one from the aminopterin sensitive, myeloma derived cell line NS1 used for the hybridoma fusion, and the other from the mouse spleen cell which carries the recognition site for the S5 antigen. Examination of the SDS-PAGE analysis of the S5/7/7/6 S300 fraction shown in fig. 3 shows that there is also molecular weight heterogeneity of the light chains in this fraction, which supports this hypothesis. The light chain with the higher molecular weight corresponds to that produced by the P3 cell line (see below). Three charge variants can arise in isoelectric focusing because of the combinatorial possibilities: 2 S5 light chains, 1 S5 light chain and 1 NS1

light chain and 2 NS1 light chains. The NS1 cell line is derived from the P3-X63-Ag8 cell line, and produces a light chain with the same isoelectric point as the P3 light chain intracellularly, but does not secrete it (KÖHLER et al., 1976). I therefore attempted to confirm that the S5/7/7/6 line contained light chains with the same isoelectric point as the P3 parent and assuming this was the case to find out which of the three isoelectric variants corresponds to the the antibody with both antigen specific light chains.

By reducing intact antibody in mercaptoethanol shortly before application to the focusing gels, I have been able to observe that the P3 and S5/7/7/6 antibody preparations each contain a chain which migrates to the same position in isoelectric focusing gels (not shown). It is reasonable to suppose that the reduced chains with the common isoelectric point are NS1 chains, but I have not been able to confirm unambiguously that this chain is a light chain. The problem arises because of the insolubility of the isolated light chains and the ease with which reduced immunoglobulin light chains become modified upon treatment with urea in the presence of reducing agents. Without urea the light chains (isolated from SDS gels) are insoluble, and with urea artefactual charge variants tend to arise. The production of artefactual charge variants is particularly severe when dithiothreitol rather than mercaptoethanol is used as the reducing agent. The technical problem is obviously not insuperable, but I have not had the opportunity to overcome it.

The staining intensity of the three isoelectric variants in the IEF analysis shown in fig. 11 is about equal. SDS-PAGE analysis showed approximately equal staining of each of the variant light chains. The observed 1:1:1 ratio of isoelectric variants deviates from the 1:2:1 ratio which would be expected if the H and L chains were to combine at random and suggests there may be preferential assembly (or secretion) of IgG molecules with the same L chain on each of the two Fab arms (G.KÖHLER, pers. comm., has also observed non-random

association of H and L chains). This distribution of P3 chains would mean than between 33% and 67% of the antibody secreted by the S5/7/7/6 hybridoma might be inactive, depending on the extent to which bivalent binding was important in the assay used. The proposed distribution of P3 chains would also provide a reasonable explanation for the differential behaviour of the S5/7/7/6 in IRMA and reverse IRMA experiments.

In IRMA the S5/7/7/6 and X4/8/6 antibodies gave an identical signal when used to detect antigen in solid phase bound hnRNP (cf. RISAU 1983, his fig. 8), but in reverse IRMA the binding of liquid phase hnRNP to solid phase bound S5/7/7/6 antibody was only 40% of that to solid phase bound X4/8/6 antibody (cf. RISAU 1983, his fig 14). In IRMA, even allowing for inactive chains in the S5 antibody preparation, both the S5 and the X4 antibodies would be present in excess. In the case of the S5/7/7/6 preparation at most 2 ng of antigen would be exposed to at least 30 ng of antibody with two active chains. In molar terms an 8-fold excess. The assay signal from IRMA given by both the S5 and X4 antibodies is therefore antigen limited. In contrast, in reverse IRMA, the amount of solid phase antibody limits the binding of labelled hnRNP (cf. inset to fig. 14 in RISAU 1983). Whereas potentially all solid phase bound X4/8/6 antibody would be able to antigen, at most 1/3 of the solid phase bound S5/7/7/6 antibody would be active if it were to bind bivalently to antigen, approximating the ratio of binding to X4/8/6 and S5/7/7/6 in reverse IRMA that is observed.

Most of the other antibodies used in the study of RISAU et al., did not appear to be heterogenous. Certainly the X4/8/6, T7/3/3/4, P11/6/3, Q18/5/4 and P46/6/7 appeared homogeneous in both SDS-PAGE and isoelectric focusing analyses. However, the V4/6/9/1 preparation appeared to contain two different molecular light chains (cf. fig 6 lane 7) and the IEF results indicated more than one cluster of bands (not shown). Of other antibodies examined, the

U25/1/9/1, T50/7/11 and W12/10/6 showed two different molecular weight light chains in SDS-PAGE, but gave no evidence of heterogeneity in IEF. The P12/8/6 and Z4/10/9 antibodies were homogeneous by both criteria. This gives a ratio of 5:7 between those hybridomas where at least one index of heterogeneity is available and those which appear to be homogeneous.

It is not suprising that hybridomas derived by fusion with NS1 cells should produce two light chains and secrete hybrid antibodies, indeed this was one of the criteria by which fused immunoglobulin-secreting and nonsecreting myeloma cell lines were first recognised (KÖHLER et al., 1976). It is perhaps more suprising that more than 50% of the cells do **not** secrete the NS1 light chain. One might ask whether it was a chance event that 5 out of the 7 lines used for the hnRNP study by RISAU et al., (1983) were homogeneous secretors. Possibly homogeneous secretors tend to give larger and more reliable signals during screening. Hybridomas secreting mixtures of light chains may lose light chain but continue to secrete IgG (KÖHLER 1980). The S5/7/7/6 hybridoma, however, was remarkably stable. It retained its stability during more than 10 ascites passages, and a subsequent further subcloning. Of 100 subclones screened until now none has been found which has lost the ability to secrete the NS1 light chain.

The presence of inactive variants, or of inactive Fab arms in an antibody preparation poses a number of problems. Firstly, the lower specific activity of the antibody may cause a lowering of the reverse IRMA response because only a limited amount of antibody can be bound to a finite area of solid phase, as in the case of the S5/7/7/6 preparation cited above. Secondly, the affinity of the antibody may seem poorer than it really is because a higher concentration of total IgG must be given to obtain a certain assay response than if only completely active antibody had been used. Thirdly, the NS1 antibody variants might give rise to unspecific binding or, even more undesirable, specific binding of an unwanted

specificity (ROCCA-SERRA et al., 1983) which would not arise if they were absent. Lastly they might cause problems in microinjection experiments for which high IgG concentrations are required and where solubility limits (see next section) set constraints.

This problem and selection methods for P3 light chain loss variants are discussed in detail by SPRINGER (1983).

R.3.5.3 Solubility of purified monoclonal antibodies

The maximum solubility of purified monoclonal antibodies under standard conditions varied appreciably. A final step in the purification of many of the antibodies was to precipitate them with ammonium sulphate and to dissolve and dialyse them against 1/10 diluted PBS (14 mM NaCl, 0.2 mM phosphate buffer pH 7.1). The idea behind this step was that if highly concentrated antibody concentrations were required, for instance for microinjection, they could be prepared by lyophilisation, without producing a high concentration of salt in the lyophilisate. The ammonium sulphate precipitates were usually dissolved at concentrations between 2 and 4 mg/ml, but even at these concentrations it was noticed that two antibodies (Z4/10/9 and T61) gave rise to a large precipitates upon dialysis. The amount of the Z4/10/9 antibody remaining in solution was not measured, but the maximum concentration of the T61 antibody was only 0.7 mg/ml. Increasing the salt concentration to 1M NaCl allowed the Z4/10/9 antibody to be dissolved, but neither undiluted PBS (140 mM NaCl) nor PBS adjusted to 1 M in NaCl was able to dissolve the T61 antibody. However by using a different pH (pH 6.0) it was possible to get at least 3.0 mg/ml of T61 into solution. The observation that the T61 antibody is poorly soluble in PBS has been confirmed by R.BABIEL (personal communication). The low solubility of the T61 antibody in PBS cannot be attributed directly to its high pI (8.3) because the P11/6/3 antibody,

which has an equally high pI, was soluble at a concentration of at least 4.4 mg/ml in this buffer.

That two antibodies differ in solubility by a factor of 6 despite identical pIs provides direct experimental evidence in support of the contention made in section R.3.2.1 that different monoclonal antibodies may be expected to vary greatly in their physical properties. I cannot exclude the possibility that the poor solubility of the T61 antibody is a subclass specific property, but the insolubility of the Z4/10/9 antibody cannot be ascribed to this cause. Other IgG1 antibodies (e.g. the plasmocytoma antibody MOPC21 from the P3-X63-Ag8 cell line) are still soluble at approximately 10 mg/ml in 1/10 diluted PBS.

R.3.5.4 Sensitivity of Mouse IgG to chemical alteration

It proved difficult to prepare tetramethyl rhodamine isocyanate (TRITC) conjugates of mouse antibodies (S5/7/7/6, X4/8/6, T7/3/3/4 and P11/6/3). In part this may have been due to the poor solubility of the derivatised protein, especially at the high pH of the derivatisation reaction (M.2.4.1.2 p.50). A red precipitate formed in the reaction vials. This was not simply made up of TRITC (which is poorly soluble), because only very small amounts of either protein ($O.D._{280}$) or protein conjugate ($O.D._{550}$) could be recovered from the supernatant of the reaction mixture following Sephadex chromatography. However, when the reaction was carried out in the cold, a substantial portion of the precipitate could be dissolved in PBS (pH 7.2, 140 mM) and labelled protein could be recovered from this material by gel filtration, although it was only weakly labelled (0.5 - 1.0 mole TRITC/mole IgG). Despite (or perhaps because of) the low degree of labelling the TRITC-conjugates were immunologically active and gave little non-specific background when used to stain either fixed Kc cells or Drosophila polytene chromosome squashes by direct

immunofluoresence. Difficulties were also encountered in storing the conjugates (see M.2.4.1.2).

FITC labelling of the same set of antibodies did not cause such problems, but the reactivity of different antibodies may differ considerably. Under standard labelling conditions (M.2.4.1.1. p. 48) fluorochrome/protein (f/p) ratios of about 3 (range 2.8 to 3.1) were obtained for the 3 IgG1 antibodies but the IgG2a antibody P11/6/3 was labelled at an f/p ratio of 6.

The reason why TRITC derivatives should differ from their FITC counterparts is not known, but HUDSON and HAY (1980, p. 13) have noted that (unspecified) fluorochrome conjugates of mouse IgG from polyclonal mouse antisera are unsatisfactory "because of denaturation of the antibody molecules". If denaturation simply means precipitation HUDSON and HAY may be referring to the phenomenon that I encountered.

Problems have also been encountered attempting to derivatise IgG1 (but not IgG2a) with either enzymes or ferritin using glutaraldehyde treatment (H.SCHWARZ pers. comm.). In these cases, however, loss of immunological reactivity rather than insolubilty seems to have been the cause of the problem.

The different responses of the antibodies in different antibody subclasses to FITC labelling suggests that the contention of section R.3.2.1 that mouse IgG antibodies cannot be considered to have uniform chemical and physical properties is correct. As far as derivatisation itself is concerned it is clear that optimal conditions for obtaining TRITC conjugates must still be sought. The labelling conditions for FITC must probably be customised at least for different subclasses of antibody, if not for individual antibodies, if comparably labelled conjugates are to be obtained.

R.3.4.5 Sensitivity of antibodies to proteolytic degradation

Monoclonal antibodies differ in their sensitivity to proteolytic degradation.

An example of this is shown in fig. 13, panel c in which the polypeptide pattern generated by papain digestion of the IgG3 antibody T61 and the IgG1 antibody P12/8/6 under identical conditions is compared. Under these conditions, the T61 heavy chain has been almost completely digested, whereas a considerable fraction of the P12/8/6 heavy chain remains intact (compare lanes 9 and 11). Furthermore, in addition to the Fc fragment of the heavy chain (indicated in panel c) and the Fab heavy chain fragment (comigrates with light chain), the T61 digest contains two additional fragments, one with a molecular weight intermediate between the Fc fragment and light chain and a doublet with a molecular weight < 21 kD (fig. 13, lane 9). These are presumably further degradation products of the Fc fragment. They indicate that the susceptibility of the T61 antibody to papain may be both qualitatively and quantitatively different from that of the P12/8/6 antibody. It is however possible to obtain intact Fabs from the T61 antibody by using very short digestion times (not shown).

I cannot tell from my data whether the difference is in susceptibility is subclass specific or antibody specific. Other investigators have reported considerable variation in sensitivity between individual IgG1 molecules (PARHAM et al., 1982; BIRCHMAIER pers. comm.).

Differential protease sensitivity may also be responsible for the appearance of a heavy chain derived polypeptide component with a molecular weight of about 40 kD which is present in most P11/6/3 preparations. This component can be seen, very lightly stained, in fig. 6 and more prominently stained in fig. 11. I strongly suspect that this component is caused by a serum protease. Almost all the heavy chain present in ascites serum which had been stored for 18 months at -20°C

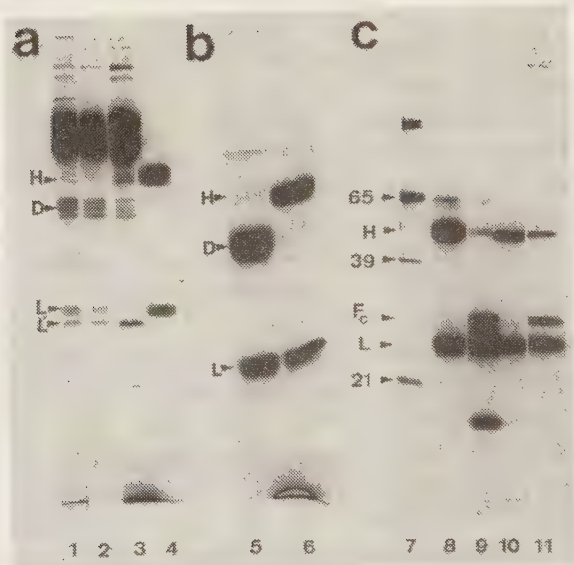

Fig. 13 **Proteolytic degradation of monoclonal antibodies.**
SDS-PAGE in 10-17% gels. Each of panels **a**, **b** and **c** is from a separate gel.
Panel a **Degraded (?) heavy chains in aged P11/6/3 ascites sera**
Lanes 1-3: 0.5 µl ascites sera (5 µl of 1/10 diluted ascites) from the 15th, 19th and 20th ascites passage (cf. table 10) of the hybridoma P11/6/3. Lane 4: purified P11/6/3 (25 µg).
Panel b **Degraded (?) P11/6/3 purified on Protein A Sepharose**
Lane 5: Preparation from aged ascites (40 µg). Lane 6: Intact P11/6/3 from an earlier preparation (25 µg) in the same gel for comparison with lane 5.
Panel c **Papain digestion under standard conditions (M.2.4.3).**
Lane 7: molecular weight markers (cf. fig. 10). Lane 8, T61 before digestion (40 µg protein). Lane 9: T61 after 4 hours digestion (40 µg protein). Lane 9: P12/8/6 before digestion (20 µg protein). Lane 10: P12/8/6 after 4 hours digestion (20 µg protein).
Key to symbols: H, IgG heavy chain; L (L'), IgG light chain (degraded?); D, degraded (?) heavy chain; Fc, Fc fragment of heavy chain; numbers, M.W. of markers in kD.

was present in this form (fig. 13, lanes 1-3) and in these sera the molecular weight of the light chain may also have altered. Despite the presumed degradation, immunologically active antibody could still be isolated from such sera by protein A affinity chromatography (fig. 13, lane 5).

It is formally possible that somatic mutation could have given rise to cells secreting an antibody molecule from which part of the heavy chain was deleted. Some plasmocytoma variants are known which produce IgGs of aberrant molecular weight (MILSTEIN et al., 1976). This could be tested by bringing frozen cells from into ascites culture and analysing the sera directly by SDS-PAGE. I have already attempted to examine the molecular weight of the heavy chain secreted by cells cloned from the ascites the analysis of whose serum is shown in fig. 13 panel a, but the concentration of antibody was too low to be seen by directly staining with coomassie blue, and radioactive labelling (M.2.2.2 p. 37) would be required to distinguish secreted protein from serum components in the culture medium.

Assuming that the altered molecular weight of the P11 in the ascites sera is caused by a serum protease, the question then arises whether protease susceptibility is subclass or antibody specific. The only other IgG2a antibody which was purified (Q18/5/4) showed no sign of lower molecular heavy chain fragments (cf fig. 6), but serum from this hybridoma was processed a much shorter time (approx. 1 month) after harvest than the P11/6/3 sera, so this comparison may not be valid. Ascites sera containing IgG1 certainly never showed any comparable degradation, even after long periods of storage, although a small amount of degradation was sometimes found in the purified antibody (cf. SDS-PAGE of P12/8/6 in fig. 8 but compare with fig. 10).

The differences in susceptibility to papain and to a presumed serum protease provide a practical example of the need to treat monoclonal antibodies in a discriminating fashion, if full use is to be made of them. The differences are presumably a consequence of the physical and chemical variation between antibodies which one might expect a priori (cf. R.3.2.1).

R.4 Purification of rabbit anti-H1 antibodies

This chapter describes the results a procedure for obtaining monospecific anti-H1 antibody from rabbit serum by affinity chromatography. Attention is drawn to possible losses of anti-H1 antibody on ion-exchange columns if IgG is prepurified from serum prior to affinity chromatography. The isoelectric point distribution of the purified anti-H1 is described and discussed.

R.4.1 Affinity Purification

Affinity chromatography on H1-Sepharose using very high ionic strength $MgCl_2$ (2.5M or 4.0M) to elute specifically bound antibody proved an effective method for purifying monospecific anti-H1 antibodies.

Fig. 14 shows the 280nm transmission profile obtained in the eluate of an H1-sepharose affinity column to which purified IgG had been applied. Most of the IgG flowed through the column, but was devoid of anti-H1 activity. Washing the column in 1M NaCl removed a small but significant amount of $O.D._{280}$ absorbing material which did not react with H1 in a CF (complement fixation see R.6) assay. It was presumed to represent non-specifically bound antibody and was discarded. The antibody eluted with 2.5M $MgCl_2$ was highly active in both CF and IRMA assays but, as the final 280nm transmission peak in fig. 14 shows some antibody remained still more tenaciously bound to the column and could only be eluted with 4.0M $MgCl_2$. It is possible that some antibody might still have been retained by the column but no attempt was made to recover it, since it was feared that any such attempt (for instance by elution with SDS) would only have delivered inactive antibody and destroyed the column.

Table 16 summarises the results of 5 different purifications. It is noteworthy that the yield of anti-H1

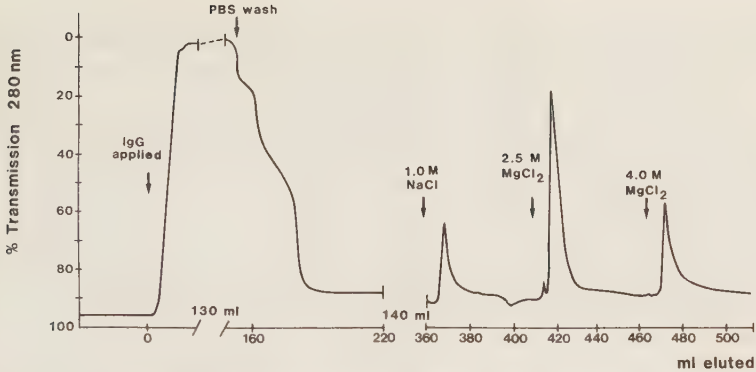

Fig. 14 **Affinity Chromatography of Rabbit anti-histone 1 antibodies on H1-Sepharose**

Light transmission at 280 nm of an H1-Sepharose column eluate. 160 ml of a solution of the purified IgG fraction (10.6 mg/ml in PBS) from the pooled sera of rabbits 401, 407 and 409 was pumped onto a 6.5 ml column of H1-Sepharose. After loading the column was washed extensively with PBS and then eluted as indicated. Transmission at 280 nm was recorded using an LKB Uvicord II.

antibody purified in this way is markedly greater from sera from which IgG was not prepurified by means of DEAE chromatography (a step which was included with the idea of minimising proteolytic attack by serum proteases on the highly susceptible - see R11 - H1 molecule). This difference could be explained by differences between initial titre (cf. table 11, p.116), but it is also possible that IgG might have been lost at a previous step in the purification procedure. Unfortunately the initial titres of the sera from rabbits 401-409 were not measured, so there was no way to test this. However, during an attempt to purify anti-H1 from the sera of rabbit 142 a 90% loss of antibody activity following chromatography on a modified DEAE matrix (DEAE affi-gel blue) was observed, as table 17 shows. According to the manufacturer this matrix allows the recovery of approx. 60% of the IgG applied in low ionic strength buffer in an almost pure form in the flow-through. A large amount of IgG was found in the flow through of the column used for the chromatography of the 142 serum (semi-quantitative Ouchterlony, not shown), in

Rabbit Nr(s).	Antigen	Volume pooled sera (ml)	IgG after DEAE (mg)	IgG Conc. pooled sera (mg/ml)	Affinity pure anti-H1 (mg)	%IgG anti-H1	Mean anti-H1 titre pooled sera (ug/ml)
401+407+ 409	RNA-H1	280	1700	6.0	12	0.7	42
402	DNA-H1	160	not applicable		19	1.5-3.0[1]	119
405+406	RNA-H1sub3[2] DNA-H1sub3	168	571	3.4	5	0.9	29
408	DNA-H1	156	664	4.2	2	0.3	13
147	tRNA-H1	169	not applicable		25	1.8-3.7[1]	150

[1] on assumption sera contains between 4% and 8% IgG
[2] Histone 1 subfraction 3 (KINKADE and COLE 1966a)

Table 16 **Yields of anti-H1 purified by affinity chromatography on H1-Sepharose.**

Sera from several bleedings, and where indicated several rabbits, were pooled and after optional initial isolation of IgG (indicated) were applied to H1-sepharose column. Yield of specific anti-H1 is sum of material eluted at 2.5M and 4.0M $MgCl_2$.

agreement with this claim, but the anti-H1 activity clearly did not behave like bulk IgG.

Some of the anti-H1 was retained by a simple ionic interaction since it could be recovered by elution with 0.5M NaCl. The remaining 70% ofthe anti-H1 applied to the column could not be eluted even with 1.4M NaCl (table 17), suggesting some more complex mode of interaction. Since this column contained not only positively charged diethylaminoethyl groups

Applied to column	11.8 mg
Flow through recovery	1.9 mg
0.5 M NaCl Elution	1.6 mg
1.4 M NaCl Elution	.01 mg

Table 17 **Recovery of anti-H1 activity from the serum of rabbit 142 applied to a DEAE affi-gel blue column.**

The amount of anti-H1 activity was determined by solid phase assay calibrated using the 402 antibody preparation as a standard.

but also Cibacron blue residues it is not possble to decide which was responsible for the removing anti-H1 activity. The behavior of the anti-H1 activity serves to illustrate the danger of assuming that the bulk properties of IgG apply to all its antigen-specific subsets.

R.4.2 Isoelectric focusing analysis of monospecific anti-H1 antibody: evidence for a stereotyped immune response to this antigen

In chapter R.5 I describe and discuss the inability of anti-H1 to immunoprecipitate H1. This characteristic raised the question of whether the immune response to this particular antigen might not be monoclonal. Here I show that this is not the case.

Fig. 15 shows the isoelectric focussing pattern generated by the anti-H1 preparations listed in table 16. Staining with coomassie blue reveals that all preparations (lanes 2, 4, 5, 7, 8 and 10) have major components focusing in the pH region 5.9 to 7.3. When the gels are more heavily loaded with antibody as with the 402 and 147 preparations (lanes 8 and 10) the space between the major bands is seen to be filled with a protein smear and the range is seen to extend still further, from pH 5.5-7.9 (402) and from pH 5.0-7.9 (147), although the species at the more acidic end of the range are clearly less abundant. This suggests that these preparations contain a very large number of minor isoelectric variants in small amounts which, because of their complexity, cannot be adequately resolved in this gel system and a few more abundant variants which can be resolved. Use of the more sensitive silver staining technique (SWITZER et al., 1979; TSANG et al., 1983) reveals a similar abundance of isoelectric variants in all preparations in the region pH 5.5-8.1, (see examples in panel B lanes 4 and 7) whereas the range of variants exhibited by the 147 preparation is hardly extended in comparison to that stained by coomassie blue (panel B, lane 10). This suggests

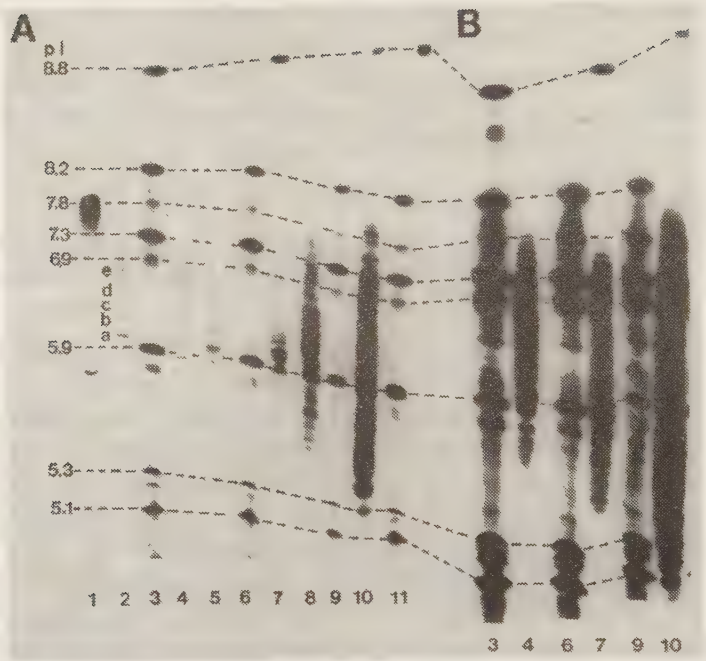

Fig. 15 **Isoelectric focusing pattern of anti-H1 polyclonal antibody preparations**

Conventional isoelectric focusing in a 1 mm thick glycerol containing 4.3% polyacrylamide gel. Servalytes focusing in the pH ranges 3-5, 5-7 and 7-9 were mixed in the ratio 1:1:2 and the gels made 14% with respect to this mixture. The gel was prefocused for 15 min at 200 V, protein samples applied to the surface of the gel halfway along its length and focussed for 6 hr at 8 Watt (500-1000V).

The polyclonal rabbit antibodies were applied to the gel as follows: lane 2, 408 (7 µg); lane 4, 401/7/9 (7 µg); lane 5, 405/6 (12 µg); lane 7, 402 4.0M H1 column eluate (12 µg); lane 8, 402 2.5M H1 column eluate (52 µg); lane 10, 147 (138 µg).

Marker proteins were as follows: lane 1, mouse monoclonal antibody P11 pI=8.1 (15 µg); lanes 3,6,9,11 1 µl of calibration mixture "protein test mixture 9" (Serva) which contained the following proteins (pIs in brackets):

ß-lactoglobulin A		(5.1)
ß-lactoglobulin B		(5.3)
conalbumin		(5.9)
myoglobin (horse)	minor band	(6.88)
	major band	(7.33)
myoglobin (whale)	minor band	(7.8)
	major band	(8.2)
ribonuclease		(8.8)

(continued on next page)

that all the preparations are highly polymorphic, presumably reflecting a vigorous polyclonal response to H1. High resolution focusing in very thin (0.1 mm) gels (not shown) confirms that the preparations are highly complex and can each be resolved into more than 50 bands. The response is clearly not monoclonal. Lane 1 shows a mouse monoclonal antibody for comparison. Although lane 1 has been purposely overloaded, the staining pattern is very different to that exhibited by the rabbit antibodies.

Although the number of minor variants is clearly very large, there appears to be some restriction in the heterogeneity of the more abundant isoelectric variants, (bands labelled a-f in the figure). Close examination of the patterns clearly reveals that there are quantitative differences between the different preparations: e.g the variant corresponding to band b is more abundant in the 405/6 and 408 preparations (lanes 2 and 5) than in the 401/7/9 preparation (lane 4), whereas the variant corresponding to band f is abundant in preparations 408 and 401/7/9 (lanes 2 and 4), but not in the 405/6 preparation (lane 5), so it is unlikely to be an artefact of the focussing system.

Nonetheless, a certain basic pattern of more strongly staining bands can be seen in all the preparations. In a separate experiment (not shown) using equal amounts of protein and non-overloaded gels the 147 and 402 preparations were also found to be qualitatively similar. A few major recurring

- -

(legend to fig. 15, continued from previous page)

the pI values correspond to those given by RIGHETTI (1983).

The isoelectric points of the marker proteins are shown on the left hand side of the gel, and the corresponding bands in different lanes connected by dashed lines to take account of the bowed focusing front. The letters a-e mark the position of bands with similar isoelectric points in the different anti-H1 preparations. In panel B the lanes have been artificially aligned with respect to the pI 7.3 band.

Panel A. Coomassie blue staining. Panel B. Examples of silver staining: marker and anti-H1 lanes 3 and 4, 6 and 7, 9 and 10.

types and a plethora of minor variants is reminiscent of the report by SCHALCH et al., (1979) on quantitative restriction of clonal responses to Staphylococcal group A polysaccharide. The pattern shown by the anti-H1 antibodies might represent a similar stereotyped response. Interestingly SCHALCH et al., (1979) showed that in their system the major antibody species showed only low affinity for antigen, whereas many (>>10) of the minor species were highly affine. As I show in section R.7.3.4 the anti-H1 preparations clearly also contained both high and low affinity components.

Idiotypes correlated with particular antigens have proved useful in defining the role of idiotype-anti-idiotype interactions in controlling the immune response (ROLAND and CAZENAVE 1981; RAJEWSKY and TAKEMORI 1983). If quantitative clonal restriction of the anti-H1 response could be confirmed this phenomenon might prove a useful additional tool to immunologists investigating network control of the immune system (JERNE 1976).

RESULTS AND DISCUSSION

PART IC

Assay Methods

FOREWORD

Three assay methods are considered in part IC: immunoprecipitation, complement fixation and solid phase assay.

Immunoprecipitation is not a sensitive technique, but it does have its uses, for example as a rapid qualitative test (Ouchterlony double immunodiffusion technique) for the success or failure of an immunisation, especially when large quantities (µg amounts) of antigen are available. It is an interesting quirk of the histone H1-anti H1 interaction that it does not lead to immunoprecipitation. Possible reasons for this are discussed in chapter R.5.

Complement fixation methodology (R.6) has now been superceded by solid phase assay procedures of improved sensitivity and convenience. However, at the time when this work was started, complement fixation was one of the most sensitive methods available. Inclusion of results obtained with this method serves the purposes of comparison (with solid phase assay) and reference (see results of chapter R.11).

Solid phase assay of nuclear extracts, immunofluoresence staining of histological preparations and "western blotting" make up a battery of tests with which the group of which I am part screens hybridoma produced antibodies for activity. The reasons for using solid phase assay are outlined at the beginning of chapters R.7 and R.8, in which the properties of the method are described.

R.5 Immunoprecipitation cannot be used for the detection of anti-histone 1 antibodies

It is not possible to use the Ouchterlony double immunodiffusion technique in agar gels for the detection of histone 1, because H1 precipitates as the result of an interaction with the agar gel itself. The bright central halo (caused by light scattering) shown in figure 16 is an example of this interaction. The symmetrical form of this precipitate is quite

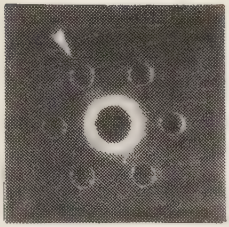

Fig. 16 **Ouchterlony double immunodiffusion of H1 and anti-H1**
The central well was filled with 20 µl of a 5 mg/ml solution of purified H1 (Worthington). 5 µl of the polyclonal monospecific anti-H1 preparation 147 at 13.8 mg/ml was placed in the peripheral well marked with the arrow. Thereafter, proceeding clockwise, serial dilutions of a factor 2 were placed in the remaining peripheral wells. The photograph shows the precipitate obtained after overnight incubation in the warm.

atypical of double immunodiffusion patterns. Control experiments (not shown), in the absence of antibody, confirmed that it was not an immunoprecipitate. The precipitate in fig. 16 is particularly striking because a high concentration of H1 (5mg/ml) has been used. At concentrations between 0.1 and 1 mg/ml, concentrations which would be quite adequate for most antigens to give reaction in a double immunodiffusion test, the precipitation reaction is much less marked.

In the experiment shown in fig. 16 there is also a faint immune reaction in addition to the non-immune precipitation, but since it was only possible to observe this reaction using a 7 mg/ml concentration of affinity purified antibody (a concentration at least 20 - 100 fold greater than the minimum amount required to give a precipitin line with other antigens), this might be attributable to a minor component in the antibody preparation reacting with some component in the H1 preparation which did not interact with agar. Proteolytic degradation products are present in almost all H1 preparations at about the 1% level (including the commercially obtained material used for this experiment) and would be good candidates for such an effect. They might lack that part of

the H1 molecule responsible for interacting with the gel matrix, while retaining the two or more antigenic determinants necessary for an an immune precipitation. The high concentration of antibody which is required could be explained if the subpopulation of antibody in the polyclonal preparation which is directed against one of these determinants only makes up a very small proportion of the total antibody population (cf. R.7.3.4).

Numerous attempts to overcome the interaction with the gel matrix, including systematic (but not exhaustive) variation of all the factors listed in table 18, proved unsuccessful. It is suprising that agarose did not remove the effect, since ALFAGEME et al., (1976) claim to have observed immunoprecipitation with H1 in this medium. It is noteworthy that their report is so worded that any positive reference to immunoprecipitation with their anti-H1 antibody is avoided and that only a lack of crossreaction of this sera with other histones is noted. In their fig. 2 ALFAGEME et al. (1976) show a photograph of a serum against another nuclear protein which shows weak crossreaction with a component from a well containing their H1 preparation. However, as their own PAGE analysis shows this H1 preparation contains a number of other polypeptides in addition to H1.

CONDITION VARIED
Composition of gel (Agar/agarose/gelatin)
Amounts of antigen and antibody (6 - 200 ug)
Monovalant cations (0 - 2M)
Divalent cations (Mg^{2+} 0 - 100 mM)
Presence or absence of detergents (Tween/Triton/PEG)
5 lots of affinity-purified antibodies from different animals

Table 18 **Conditions varied in search for suppression of H1-gel matrix precipitation reaction.**

In two of the most recent reviews on the immunology of histones STOLLAR (1978) and BUSTIN (1978) are agreed that immunodiffusion methods are unsuited to the analysis both of H1 and of histones in general. This conclusion is supported by the argument that histones tend to form unspecific precipitates with a component of serum other than immunoglobulin, probably α_2-macroglobulin, which causes precipitin reactions with non-immune sera (STOLLAR and REZUKE 1978). Precisely such a reaction with histone H4 is reported by ALFAGEME in the study cited above. Although H1 forms precipitates with α_2-macroglobulin less well than the other histones, (STOLLAR and REZUKE 1978), it does form precipitates with bovine serum albumin (BSA, see below), and because the structure and isoelectric point of serum albumins are conserved between mammalian species, it is reasonable to suspect that such an interaction could occur with the large quantities of albumin present in the sera of rabbits and other animals used to provide immune sera. An interaction of H1 with a serum component is therefore feasible. Such an explanation can however be ruled out as the cause of precipitates such as those in the central ring shown in fig 16: the IgG used for these immunodiffusion experiments was highly purified. An interaction of an antigen with the gel matrix is unusual, so there is no reason why other investigators should include a control to test for it.

An additional reason why such an interaction may have gone undetected until now is that a more usual protocol for the Ouchterlony double immunodiffusion test than that shown in fig. 16 is to place antiserum in the central well and antigen in peripheral ones. Under these conditions low concentrations of antigen sometimes cause precipitates to form very close to the edges of the peripheral wells. Even an experienced investigator could not easily distinguish these from a symmetrical precipitate caused by interaction with the gel.

Attempts to form immunoprecipitates in solution using highly pure affinity purified anti-H1 antibody (see R.4) and standard turbimetric methods for their detection were equally unsuccessful. An attempt to test the possibility that the highly charged H1 molecule might be immunoprecipitable when complexed (and charge-neutralised) by nucleic acids was frustrated because the complexes formed between H1 and tRNA (used as a convenient source of nucleic acid) themselves precipitated. In the course of these experiments histone 1 was also found to form precipitates when incubated with the acidic protein BSA (pI 4.7) in the appropriate ratio.

Thus, although lack of immunoprecipitate formation in gels appears to have a trivial explanation (interaction with the gel matrix) the results of the solution experiments are not so easily explained and suggest that there may indeed be some fundamental peculiarity associated either with the anti-H1 : H1 complexes themselves, or with the composition of the anti-H1 preparations.

Two mechanisms can cause an animal to produce an antiserum which fails to give an immunoprecipitate, although the serum contains antibodies which bind to antigen:

1) a monoclonal immune response or

2) a polyclonal response against only a single determinant on an antigen.

In either case no network formation between antigen molecules would then be possible unless the antigen carried repetetive determinants.

There are some precedents for monoclonal responses. Certain inbred strains of mice make nearly monoclonal responses, of a particular idiotype, against certain haptens e.g. Balb/c mice against phosphorylcholine (TEPC15 idiotype COSENZA and KÖHLER 1972), A/J mice against arsonate (CAPRA et al., 1975) and C57BL6 and CBA mice against (4-hydroxy-3-nitrophenyl)acetyl (McMICHAEL et al. 1975). Selectively bred

rabbits can also produce a monoclonal response, when hyper-immunised with streptococcal group A polysaccharide whereby a single isoelecric IgG variant (with different isoelctric points in different rabbits) makes up 90% of the serum IgG (BRAUN et al., 1973). Closer scrutiny of hyperimmune responses in both mice (RETH et al., 1978) and rabbits (SCHALCH et al., 1979) reveals that monoclonality is quantitative rather than qualitative and that the affinity of the major monoclonal antibody for antigen is weak.

The possibility of a monoclonal response to H1 can be eliminated: isoelectric focussing analysis of the anti-H1 preparations reveals that they are at least oligoclonal (R.4.2). IRMA and ELISA analysis suggests that they contain components with different affinities for the antigen (R.7.3.4) and there are differences between anti-H1 preparations from different animals in their crossreactivity with H1 molecules from other species. Taken together these observations suggest individual preparations must be able to react with spatially separated antigenic sites. Nonetheless, despite these functional differences between anti-H1 preparations, the small number of isolectric variants (R.4.2) suggests that the number of sites might be limited.

Another possible explanation is that charge repulsion between the highly positively charged histone 1 molecules hinder the formation of large aggregates, but then it is suprising that increasing the ionic strength did not suppress this effect. In conclusion, the inability of anti-H1 antibodies to immunoprecipitate H1 remains a mystery.

R.6 Quantitative micro-complement fixation assays

Complement is the name given to a set of 9 different proteins present in the sera of most mammals which is responsible for the lysis of foreign cells (e.g. bacteria). The proteins react sequentially with each other in a complicated cascade process (see REID 1983 for a recent review). The complement pathway is triggered by antigen-antibody complexes which bind to a subunit (C1q) of the first component of complement (C1). The C1 to which the immune complexes are bound is inactivated as the result of subsequent steps in the pathway (REID and PORTER 1981).

The complement fixation assay uses the inactivation of C1 in a serum with high complement activity as a measure of the amount of antigen-antibody complexes in the sample to be assayed. Active C1 is measured by the ability of the complement-containing sample to lyse Sheep erythrocytes (SRBC - Sheep Red Blood Cells) to whose surface a standard amount of anti-SRBC antibody has been bound. When the amount of complement added to the assay system is carefully adjusted, so that it is just sufficient to lyse all the SRBC in the assay, then **inhibition** of lysis provides a sensitive assay for antigen-antibody complexes (OSLER and HILL 1955, WASSERMAN and LEVINE 1961, LEVINE 1978). N.B. The greater the **inhibition of lysis** the greater the amount of antigen-antibody complexes in the assay. In the standard form of the assay, described below (R.6.1), the extent of lysis is monitored by pelleting the red blood cells and measuring haemoglobin release spectrophotometrically. By providing antigen in excess unknown amounts of antibody can be assayed and, vice versa, unknown amounts of antigen can be determined.

R.6.1 Haemoglobin release assay

Fig. 17 shows an example of titrations with 3 different amounts of an affinity purified monospecific anti-H1 antibody with different amounts of histone 1.

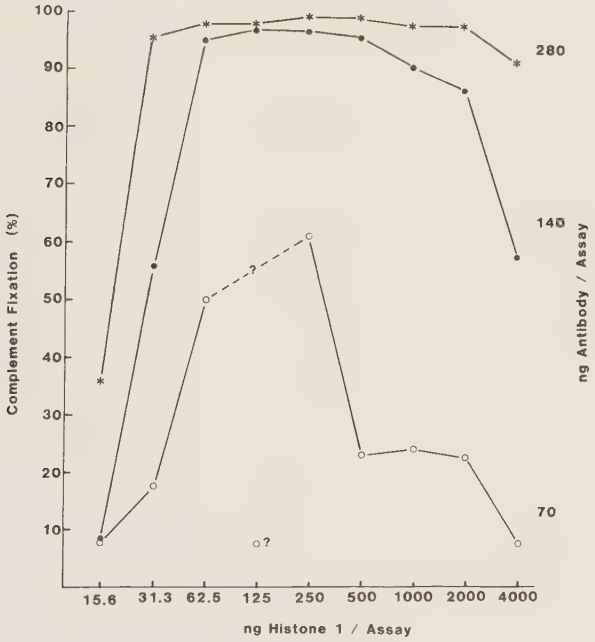

Fig. 17 **Titration of antibody and antigen by complement fixation.** A concentrated stock solution of affinity purified anti-H1 derived from rabbit 402 (cf. R.4.1) was diluted with KBR buffer (M.2.5.1.2) to 70, 140 and 280 ng anti-H1 in 100 µl. 100 µl of each dilution was mixed with the amounts of H1 indicated on the abscissa and 250 µl of a 1/250 dilution in KBR of a complement containing guinea pig serum. After adjusting the volume to 1 ml with KBR buffer the assay mixture was incubated for 18 hr at 4°C. Complement fixation was assayed by the inhibition of SRBC lysis as described in M.2.5.1.2. The curves corresponding to each of the three concentrations of antibody are indicated by the numbers (ng of antibody present in each assay tube) on the right-hand side of the figure. A logarithmic scale has been employed for the abscissa.

The bell-shaped curve with the flattened middle region is characteristic of this assay (WASSERMAN and LEVINE 1961, SLUYSER and BUSTIN 1974, MIHALAKIS et al., 1976, BUSTIN 1978, LEVINE 1978, STOLLAR 1978). Notice that this assay allows the detection of sub-microgram amounts of both antigen and antibody.

Despite its sensitivity, this method has several disadvantages. Both the amount of complement and the amount of anti-SRBC antibody must be carefully titrated to realise the full sensitivity of the test. Once the SRBC have been sensitised, their susceptibility to complement remains constant for only 2 to 3 days. Untreated SRBC can be stored for several weeks but their susceptibility to lysis also changes slightly with time, so that the titrations must be repeated frequently. In addition the complement system is sensitive to a number of reagents and serum components, which means that the assay must be modified for use with e.g hybridoma supernatants. Adequate controls for non-specific inactivation of complement must be included in every test. One further disadvantage is that the keeping qualities and susceptibility to lysis of SRBC from different sheep can vary widely (unpublished observations).

The anti-H1 titre of antisera was arbitrarily defined as the lowest dilution (highest concentration) of antisera (heated treated to inactivate any complement it might itself contain see M.2.5.1.2.1) which gave no response (< 10% Complement Fixation) in the standard assay using a standard amount of H1 (250 ng).

R.6.2 ^{51}Cr release microcomplement fixation assay

The sensitivity of the standard microcomplement fixation assay is limited by the absorbtion coefficient of Haemoglobin. A $\Delta O.D._{413}$ of about 0.05 or about 10% complement fixation (CF) is the smallest change that could be reliably measured using

standard laboratory equipment. This required the use of 10^8 cells per assay. Under conditions which increased the sensitivity of the assay to H1 (see R.11.2). The minimum amount of complement necessary to lyse this number of cells set empirically determined limits of about 2ng antigen - 90 femtomoles, 6.10^{10} molecules or about 10ng antibody - 70 fmoles, 4.10^{10} molecules - on the minimum amount that could reliably be detected. These values are similar to those obtained by other investigators using a variety of antigen-antibody systems (see Table 5B.4 in LEVINE 1978).

^{51}Cr loaded cells are used by immunologists for a variety of cytotoxicity tests which require very small numbers of cells and are therefore very sensitive (BRUNNER et al., 1968, GRABSTEIN 1980). The antibody titration curves shown in fig. 18 show that it is possible to adapt these techniques for use in the complement fixation assay. Only 2.10^6 cells were used for the titration shown in fig. 18, and a 10 fold increase in sensitivity was achieved. An arbitrary amount of antigen, rather than the amount giving the maximal antibody response (cf. fig. 17), was used in this set of experiments and was very probably too high. It is therefore possible that the 50-fold increase in sensitivity expected from the reduced number of erythrocytes used in the assay, could have been achieved had this assay system been developed further. Assuming that the proportionate increase in sensitivity achieved with ^{51}Cr can be extrapolated to the assay used in section R.11.2) then this would give the assay the ability to detect approx. 10^9 molecules of antigen or antibody. However, solid phase assay is not only more sensitive (by approximately a factor 10, see R.7), but is also more convenient, since it does not require reagents to be regularly recalibrated. As the advantages and the convenience of solid phase assay techniques became apparent complement fixation methodology was abandoned.

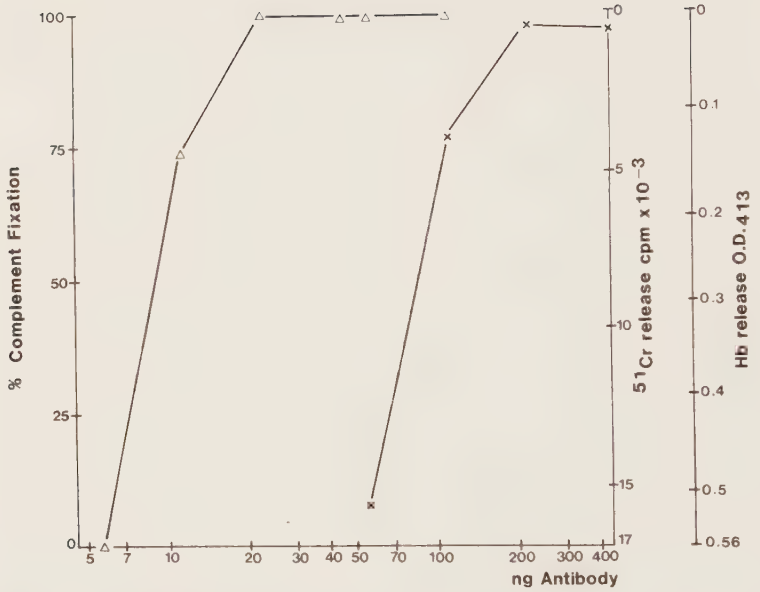

Fig. 18 **Increased sensitivity of complement fixation assay for detection of antibody using ^{51}Cr release method.**

"Hb release" curve shows complement fixation measured by inhibition of haemoglobin release from 10^8 SRBC. "^{51}Cr release" curve shows complement fixation measured by inhibition of ^{51}Cr release from 2. 10^6 ^{51}Cr loaded SRBC. The amount of antibody used to obtain each experimental point is indicated on the abscissa (N.B. logarithmic scale). 250 ng H1 was used as antigen in each assay. Incubation conditions were identical until the SRBC were added. The actual measured quantities from which the extent of complement fixation was computed are indicated on the right hand side of the figure ($O.D._{413}$, cpm ^{51}Cr). The maximum amount of ^{51}Cr release in this particular assay (17,000 cpm) is less than that usually obtained (<4. 10^4 cpm), because the loaded red cells had aged.

R.7 Solid Phase Immunoassays

Antibodies are specific reagents for the detection, localisation and, in principle, quantitation of the antigens they recognise. To fully realise the usefulness of these properties it is often neccessary to screen large numbers (hundreds) of samples either for antibody or antigen content. Handling steps must be kept to a minimum if such large scale tests are to be performed efficiently. One way of doing this, for which materials are commercially available, is to use plastic plates ("microtitre" plates) into which is set a regular array of 96 depressions or "wells". Each well has a volume of about 300 µl and can serve as an individual "test tube" in which antigen-antibody binding reactions can be carried out. The absorbtive properties of the plastic material (plates made out of different plastics are available) are used to bind the antigen to a solid phase. After subsequent incubation with antibody it becomes an easy matter to remove antibody free in solution from that bound to the solid-phase. The bound antibody may then be visualised by means of an appropriately labelled detection reagent, such as protein A or a second antibody (anti-antibody), hereinafter referred to as **detector.** Relative amounts of either antibody or antigen may be measured by holding one of the reactants constant and titrating the other. By using multichannel pipettes the number of individual pipetting steps is kept to a minimum and it is easily possible for a single person to process 1000 tests per day. The primary problem then becomes that of evaluating the data thus produced, rather than generating it. However, with the advent of cheap machines for the aquisition, rapidly accessible storage and processing of data, such problems are no longer insuperable.

The solid phase immunoassays used in our laboratories were developed empirically. They were used because they worked. They gave a signal that clearly distiguished between

immune and non-immune sera or between the presence or absence of specific antibody in hybridoma supernatants (SAUMWEBER et al., 1980; SAUMWEBER 1980) and allowed sufficient tests to be made per unit time to satisfy the requirements of our test program.

In this chapter I detail some characteristics (sensitivity, efficiency and capacity) of solid phase assays and indicate the minimum amount of antigen required for an antibody against it to be detected using this assay technique.

Two different techniques can be used for detecting solid-phase antibody-antigen complexes. In the IRMA assay (for nomenclature see R.7.1) the antibody detector is labelled with ^{125}I and the amount of bound label is counted. In the ELISA assay the antibody detector is labelled with an enzyme which converts a colourless substrate to a coloured product and the amount of bound antibody is estimated by measuring the rate of colour production. I should make it clear at the outset that, under the conditions used in our laboratory, the two methods are complementary. To achieve maximum IRMA sensitivity the detection reagent is labelled to very high specific activity (approx. 1 $\mu Ci\ pmole^{-1}$). In consequence the range of measurement with the detection reagent is limited by the danger of contamination and considerations of safety and legality (Strahlenshutzgesetz). For example, when the wells are coated with pure antigen, an input of approximately 50 pmole, about $3\ 10^8$ cpm, of detector **per well** would be required to saturate the maximum possible amount of antibody which could bind to the surface of the well (see R.7.3.5), i.e. it would require >2mCi of labelled reagent to assay all 96 wells of a microtiter plate for maximum detector binding. As a practical upper limit, at most $2.5\ 10^5$ cpm per well was input. The necessity of working with such limiting concentrations of detection reagent sets limits on the range and sensitivity of the assay which are not intrinsic to the method. It would, of course, be possible to reduce the specific activity of the

detector to alter the range antigen measurable, but only at the cost of reducing sensitivity. Safety and contamination constraints do not apply to the concentrations of immunological reagents which can be used in the ELISA, thus the range of values which it can be used to measure is greater (R.7.3.4.2, R.7.3.1.2.2), although its sensitivity in my hands was slightly less than that of IRMA (cf. R.7.3.1.2.7). Furthermore, some of the surface properties of the plates usually used for each kind of assay (PVC for IRMA, polystyrene for ELISA) are clearly different (cf. R.7.3.5.1) and this might also affect the outcome of each of the assays, especially when mixtures of antigens are applied to the plates. Finally, for the reasons explained in R.7.3.1.2.7 the specific activity of an enzyme coupled detector is not conveniently determined. In contrast, the the specific activity of a radiolabelled antibody can be easily estimated, allowing determination of absolute numbers of bound detector molecules.

The maximum sensitivity of the assay (i.e. the minimum amount of antigen which can be detected) will be determined by a number of interdependent factors.

Among those which were investigated were:

1) the ability to discriminate specific antibody binding from non-specific binding or other sources of background counts (i.e. the signal to noise ratio, R.7.3.1.2.3, R.7.4.4);

2) the specific activity of the antibody detection reagent (R.7.3.2.1, R.7.3.2.2);

3) the extent to which the antibody-antigen reaction has been completed, important when a liquid-phase reactant is limiting (R.7.2.1.4, R.7.2.3, R.7.3.3);

4) the capacity of the plate, important when an antigen comprises only a small proportion of the protein applied to the solid phase (R.7.3.5.2, R.7.4.3);

5) the efficiency of the various binding reactions i.e. the measured stoichiometry of the reaction (R.7.4.4.2)

6) selective binding of an antigen to the solid phase or its converse, selective exclusion from binding (R.7.4.1, R.8.2).

Section R.7.3 describes estimates of the magnitude and importance of these factors based mainly on the results of model binding experiments using the purified protein calf-thymus H1 as antigen together with its corresponding mono-specific (polyclonal) antibody. Analagous experiments using purified mouse IgGs as antigen are reported in chapter R.8. These model substances were in many ways non-ideal (see R.7.2.4 and R.7.2.5), but were used because they were convenient and readily available materials, and sufficed to characterise some of the grosser features of solid-phase methodology.

Section R.7.4 describes some results obtained using monoclonal antibodies and antigen mixtures and attempts to interpret them in the light of the results obtained from the model experiments.

The two immediately following sections explain the nomenclature of assay techniques (R.7.1) and theoretical basis of antibody-antigen binding (R.7.2).

R.7.1 Nomenclature of assay techniques (IRMA, RIA, ELISA)

Although one might imagine that the connotation radio-immunoassay (RIA) is neutral, in the biochemical and immunological literature this term has been used almost exclusively, until recently, to designate competition assays of the the type pioneered by YALOW and BERSON (1960) (see e.g. PARKER 1976, COOPER 1977, HUNTER 1978). In this form of assay an unknown amount of an antigen is estimated by means of the extent to which it competes with a small amount of labelled antigen for binding to specific antibody (often attached to a solid phase). Quantitation can be achieved by calibrating the inhibition of label binding with known amounts of unlabelled

antigen. This sort of test requires the use of highly purified antigen in solution and increasing the amount of antigen used in the assay results in decreasing amounts of bound counts. Competitive RIA assay is very widely used, especially for clinical applications.

The radioassay referred to in this work which is used to estimate antigen is very different: the antigen is impure and bound to the solid phase; increasing the amount of antigen used in the assay increases the amounts of counts bound. Although our group has used the term RIA to describe assays using solid-phase bound antigen (SAUMWEBER et al., 1980, RISAU et al., 1981), to avoid confusion between the two different forms of assay, it would seem prudent in future to use the designation IRMA, an acronym for ImmunoRadioMetric Assay (MILES and HALES 1968), for this assay. IRMA is a generic term for assays in which the amount of antibody bound to antigen is counted (THOMPSON 1982).

ELISA stands for EnzymeLinkedImmunoSorbent Assay and although many different forms of ELISA are practised (see e.g. SEDGWICK and HOLT 1983, LEMS-VAN KAN et al., 1983, KENDALL et al., 1983, ALI and ALI 1983), among them assays similar to the ones we use (CARROLL and STOLLAR 1983), the connotation has not acquired such a highly restricted meaning as the one accorded to "RIA".

R.7.2 Theoretical aspects of antigen-antibody complex formation

This section is included to show that, while the binding behaviour of antibodies to solid-phase adsorbed antigen can be described, formally, by a set of equations, there is no single paradigm to explain the relationship of the terms "activity", "concentration" and "affinity", used to describe a homogeneous phase (solution) reaction, to their counterparts in a heterogenous phase reaction. Use of these terms is necessary to be able to use the formalism, which can be shown

experimentally (R.7.4.4, R.7.4.4.2) to be appropriate. However, depending on how the terms noted above are defined, numerical values for binding constants very different from those obtained for homogeneous phase reactions may be obtained.

Obviously, in principle, quantitation of antigen should be possible by simply exposing antigen to excess antibody and counting the number of bound antibodies. However, to achieve this goal a number of important practical problems must be solved. How does one know that antibody is saturating antigen, especially if the affinity of the antibody for its antigen is weak? If the antibody crossreacts with low affinity to other (non-specific) sites (BERZOFSKY and SCHECHTER 1981; FAGRAEUS et al. 1983), then it may never be possible to use sufficiently high concentrations of antibody to achieve saturation of specific sites without interference from the non-specific binding signal. There may be reasons (e.g. expense, degree of labelling) why saturating concentrations of antibody cannot practicably be employed. In such cases it would be useful to be able to extrapolate results obtained using non-saturating antibody concentrations, but then it is necessary to know the relationship between antibody binding and concentration. Equations for describing the binding of monovalent ligands to proteins in a homogeneous solution have been derived (SCATCHARD 1949, KLOTZ 1953) and the effect of antibody affinity on complex formation under these conditions is described in section R.7.2.1. The restriction of one of the reacting components to a rigid surface raises a set of questions about how this might affect quantitative binding. Will binding of one or other of the reactants to a solid phase affect the equilibrium binding position? How will binding be affected by the valence of antigen or antibody or both? How might heterogeneity of antigen or antibody affect the relationship? What effects might excluded volume (i.e when a previously bound molecule excludes or hinders the binding of subsequent molecules) have on solid phase binding? Will the

time course of the binding reaction be affected?

Consideration of the reported variation in the intrinsic affinity of antibody for antigen (R.7.2.1.2) and of valence and solid phase effects (R.7.2.2.1, R.7.2.2.3 and R.7.2.3.4) shows that, depending on these factors, and on the concentration of antigen, that antibody concentrations differing by several orders of magnitude may be required for the production of detectable immune complexes. Given this degree of variation it is important to choose the appropriate description of an antigen-antibody system to avoid making conclusions about amounts of antigen in the system which are seriously in error. In what follows I have tried to find the explicit expressions describing the relationships between the concentrations of reactants and complex, making various assumptions about the nature of the antibody-antigen interaction, but since an exact solution of some of the theoretical problems is still outstanding this is not always possible.

Sections R.7.2.4 and R.7.2.5 outline some of the reasons, in part arising from inadequate theory, which prevent a complete analysis of the experimental binding results described in section R.7.3, even though these were obtained using highly purified antibody and antigen.

R.7.2.1 General properties of immune complex formation in a homogeneous phase

R.7.2.1.1 Microscopic rate constant

The most easily understandable theoretical element of antigen-antibody complex formation is that of the microscopic rate constant (CALVERT et al., 1979) or "site constant" (SINGER 1965) K_{eq}, which describes the equilibrium reached in a closed system between antibody combining sites (Ab_{cs}) and

antigenic determinants (Ag_d) associating with second order rate constant k_{+1} and dissociating with first order rate constant k_{-1}.

$$Ab_{cs} + Ag_d \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Ab_{cs}{\cdot}Ag_d \qquad \text{Reaction Scheme 1}$$

Denoting activities of components by using square brackets, complexes ($Ab_{cs}{\cdot}Ag_d$) by B and the total concentration of each reactant by the prefix T, then by the law of mass action the equilibrium or binding constant K_{eq} for each site is given by

$$K_{eq} = \frac{k_{+1}}{k_{-1}} = \frac{[B]}{([TAb_{cs}]-[B])([TAg_d]-[B])} \qquad \text{Eq. (1)}$$

For reactants in dilute solution (i.e. in a homogeneous phase), activity, a measure of the probability of reaction, is closely approximated by using concentration in terms of molarities.

The above expression Eq. (1) holds if either antigen or antibody (but not both - see below) is multivalent and the intrinsic affinity of each valence is equivalent (SCATCHARD 1949; KLOTZ 1953). In such cases the molecular concentrations must be multiplied by the valence to arrive at the site concentration.

For experimental determinations of K_{eq} linear transfomations of Eq. (1) allow graphical representation and more accurate regression analysis of binding data (FAZEKAS de St.GROTH 1979).

e.g. using the transformation first employed by SCATCHARD (1949), for an antibody (Ab) of valence n

$$\frac{[B]}{([TAg_d]-[B])} = -K_{eq}[B] + K_{eq}n[TAb] \qquad \text{Eq. (2)}$$

By varying the total amount of antigen in the system, measuring amounts of bound ([B]) and free antigen ($[TAg_d]-[B]$)

and plotting the bound/free ratio against the bound concentration, a line of slope $-K_{eq}$, with x intercept n[TAb] and y intercept $K_{eq}n$[TAb]. Deviations from linearity of such "Scatchard" plots provide a useful indication of antibody heterogeneity or of interaction between binding sites on a multivalent antibody. K_{eq} can, of course, also be estimated from the concentration of bound and unbound antibody. However, if the antibody is multivalent, it is first necessary to derive the fraction of bound combining **sites** from the fraction of bound **molecules** (see M.7.4).

If K_{eq} is very large unbound material will only be present at very low concentrations and may be difficult to measure accurately. Direct estimation of k_{+1} and k_{-1} (initial rate measurements) may then be preferable way to obtain a value for K_{eq} (MASON and WILLIAMS 1980, PARHAM et al. 1982).

Binding relationships of the type represented by Eq. (2) are useful for describing the binding of:

a) antibodies with haptens (intrinsically non-antigenic low M.W. substances against which antibodies can be raised by immunising with hapten-macromolecule conjugates);

b) monoclonal antibodies to soluble macromolecules with a single antigenic determinant;

c) antibodies to determinants which form part of a surface, such as viruses, ribosomes, cells, antigen adsorbed to a solid phase providing that the mean distance between determinants is sufficiently great to exclude divalent binding of antibody (FAZEKAS deSt.GROTH 1979);

d) Fab or Fab' fragments to multivalent antigen or to antigens bound to a surface.

R.7.2.1.2 Range of binding and rate constants for antibodies and detector molecules

In the era prior to the introduction of hybridoma technology (KÖHLER and MILSTEIN 1975) and the subsequent widespread availability of monoclonal antibodies, reliable

estimates of antibody **site binding constants** were almost entirely limited to antibody-hapten interactions (summarised for example in PARKER 1976 p. 111ff.). Such interactions can be easily measured in a homogeneous liquid phase using techniques such as equilibrium dialysis and the reaction can fully characterised by using the techniques described in the previous section. Unfortunately antibody-hapten binding is often a poor model for antibody-protein antigen interactions (cf. FAZEKAS deSt.GROTH 1979 p.12 ff. and below).

Complications arising from antibody heterogeneity and multivalent interactions (see e.g. CALVERT et al., 1979) have presumably been primary reasons for the neglect of quantitative studies of this most elementary step in antibody-protein antigen binding. The only two studies which I have been able to find which were made using polyclonal anti-protein antibodies both relied upon fluoresence polarisation techniques to circumvent the complications introduced by these factors (COLEMAN et al., 1972; LEVISON et al., 1970).

Estimates of "avidity" or "functional affinity" (a poorly defined term, compare e.g. EISEN 1966 with HOLMES and PARHAM 1983, which attempts to describe the quantitative relationship between antibodies and antigens in the formation of multivalent immune complexes) have of course been made for anti-protein antibodies. Picomolar concentrations of polypeptide hormone antigens are assayed using antibodies from high "avidity" antisera (YALOW and BERSON 1971, YALOW 1980). However, multivalence, polyclonality and the conditions affecting antibody-antigen network formation make "avidity" values dependent upon test conditions (e.g. upon homogeneous or heterogeneous phase reactions see R.7.2.2.1) and may limit the relevance of a determination to an antiserum from a particular bleeding from a particular animal. Such measurements may provide a useful relative measure of immune "strength", but they do not allow conclusions to be drawn about the elementary molecular interactions involved and may only be applicable in the context of a particular assay.

There are now about 20 determinations either of site binding constants or of rate constants of monoclonal antibodies reacting with protein antigens. These, together with data for the IgG-binding protein Protein A, are summarised in table 19.

Property	Monoclonal Antibodies (range)	(Refs)	Protein A (binding to rabbit IgG)	(Refs)
Site Constant (K_{eq}) ($l\ mol^{-1}$)	$2\ 10^7$ - $5\ 10^{10}$	(1,2,3,4,5)	$1.3\ 10^8$	(6,7,8)
Forward rate (k_{+1}) ($l\ mol^{-1}\ s^{-1}$)	$2\ 10^4$ - $2\ 10^6$	(1,2)	(approx. 10^5)	(9+)
Reverse rate (k_{-1}) (s^{-1})	10^{-3} - 10^{-5}	(1,2)	(approx. 10^{-3})	(9+)

References: 1. MASON and WILLIAMS 1980
2. PARHAM et al., 1982
3. FAZEKAS de St.GROTH 1983
4. TRUCCO and dePETRIS 1981
5. WAGENER et al., 1983a
6. JONSSON and KRONVALL 1974 } mean of these 3 estimates
7. KESSLER 1975 }
8. SANDOR and LANGONE 1981 }
9. O'KEEFE and BENNETT 1980

+ no direct measurement, rough estimate from dissociation times in 9

Table 19 **Range of reported binding constants for monoclonal antibodies binding to protein antigens and for the binding of protein A to rabbit IgG.**

Unfortunately, many of the values in table 19 were obtained by measuring the binding of antibodies to cell surfaces, because the antibodies were directed against membrane proteins. The appropriateness of using homogeneous phase binding equations to derive site binding constants for membrane binding antibodies, without redefining the terms of Eq. (1), has been cast in doubt (DELISI 1981: see also R.7.2.1.5, R.7.2.2.2 and R.7.2.3.4).

The values in table 19 compare with a range of values for K_{eq} between 10^6-10^9 M^{-1} for the binding of a variety of antigens to polyclonal antisera, measured using the fluor-

esence polarisation technique referred to at the beginning of this section (COLEMAN et al., 1972; LEVISON et al., 1970). A single forward rate estimate of 10^5 M^{-1} s^{-1} for a polyclonal antibody preparation was also obtained using this technique (LEVISON et al., 1970). The overlap between these values and those in table 19 suggests that the values are representative of most antibodies and can be used for the further dicussion of antibody binding behaviour.

I have disregarded one K_{eq} estimate of 10^{10} l mol^{-1} (by O'KEEFE and BENNETT 1980) for Protein A, because the results from the 3 cited studies are in agreement with those from binding studies using human IgG (KRONVALL et al. 1970b, MYHRE and KRONVALL 1980) for which it is known, from competition studies, (LANGONE 1978, 1982a) that protein A has a similar affinity.

Table 19 shows, as pointed out by PARHAM et al., (1982), that variation in both forward and reverse rates contributes to the observed variation in K_{eq}. In particular the lowest forward rates fall far below the limits set by collisional frequency (approximately 10^8 l mol^{-1} s^{-1} for proteins, VON SMOLUCHOWSKI 1916, KÜPPERS, 1983 p. 229), meaning that there is a very high activation energy associated with some antigen-antibody interactions (as suggested by LEVISON et al., 1970). This observation contrasts with the results obtained by studying antibody-hapten interactions. These studies indicate roughly two classes of immune reaction (DEMBO et al. 1979). One class is characterised by forward rates close to the limit set by collisional frequency (10^7 - 10^9 M^{-1} s^{-1}, i.e the reaction is diffusion limited) and variations in K_{eq} are mainly controlled by variation in the dissociation rate constant (k_{-1}), whose value is seldom lower than 1 s^{-1} (FROESE 1968, DAY et al., 1972). The antibodies in the other class have properties similar to those of the anti-protein ones: the forward rates lie well below ($<10^{-7}$ M^{-1} s^{-1}) the limit set by diffusion and the reverse rates are very low (e.g. 10^{-3} - 10^{-5} s^{-1}) (PECHT and LANCET 1976). Unfortunately, the first

class was overrepresented in early studies (until about 1975), with the result that diffusion limited reaction rates were assumed by many authors to be general for antibody-hapten interactions (see e.g PARKER 1976).

One practical consequence of this variation is that the time course of (protein-antigen)-antibody interactions, using different (monoclonal) antibodies, may be very different, even though the equilibrium position which will eventually be reached may be the same (see R.7.2.1.4). The dissociation rate of some monoclonal antibodies is really remarkably low, $t_{1/2}$ varying between approximately 12 minutes ($k_{-1} = 10^{-3}$) and 20 hr ($k_{-1} = 10^{-5}$).

An additional source of variation between monoclonal antibodies is their sensitivity to temperature. The dissociation rate of at least one monoclonal has been shown to change by an order of magnitude as a result of a 20°C change in temperature, but the properties of other monoclonals are essentially unaffected by temperature changes of this magnitude (MASON and WILLIAMS 1980, see also R.7.4.7).

Some further practical implications of the data shown in table 19 are examined in the immediately following section.

R.7.2.1.3 Predicted binding equilibria under ideal homogeneous phase conditions

On the basis of the range of affinities in table 19, I show in this section that the site binding constant of an antibody can be an important factor determining the sensitivity with which immune complexes can be detected, especially if antibody is highly dilute ($<10^{-10}$ M). I show the theoretical limits to sensitivity.

For the purposes of demonstration, I ignore the practical problems of separating bound and free antibody and consider what would happen if antibody were to combine with antigen under ideal homogeneous phase conditions. To do this I have computed the amount of immune complex formation at different

values of K_{eq} and at two different concentrations of antibody, assuming that the maximum amount of antigen containing protein which can be used in the reaction is 800 ng and that the reaction volume is 0.1 ml. These amounts have been chosen to be comparable with those used in solid phase immunoassay (cf. R.7.4.3). Computations have been performed for two concentrations of antibody, $7\ 10^{-9}$ M (1 µg/ml) and $2\ 10^{-10}$ M (30 ng/ml), which correspond to the concentrations at which monoclonal antibody and radiolabelled detector respectively are employed in IRMA.

Fig. 19 shows how the intrinsic affinity and the absolute concentration of an antibody interact to determine the efficiency with which an antigen can be detected. At the higher concentration of antibody (solid line in panels a,b,c), a K_{eq} $>10^{9}$ is sufficient to give rise to near saturating complex formation (>90%), and as comparison of panels a), b) and c) shows this behaviour is essentially the same irrespective of antigen concentration over the range 10^{-12} to 10^{-8} M. Conversely, even when antibody is in vast molar excess (panel a; molar ratio of antibody to antigen approx 10^{4}), if the K_{eq} of the antibody is less than 10^{8} M^{-1}, then at most 50% of the antigen will be able to bind antibody. The effect of using more dilute antibody (compare dashed and solid lines in panels a and b) is to strongly reduce the binding efficiency of all except the most affine antibodies. As panel a) shows even when there is a 100 fold molar excess of antibody a site binding constant of $>10^{10}$ M^{-1} is required if antigen is going to form immune complexes quantitatively. In other words if the K_{eq} of the detector is less than 10^{10} M^{-1} it is likely to be the factor determining the sensitivity of the immunoassay.

This conclusion is more clearly shown in fig. 20, in which the amount of detector bound is plotted as a function of antigen concentration: note the log scale of the ordinate in this figure. Panel a shows, that at these low concentrations of detector, even if antigen is in molar excess (>$2\ 10^{-10}$ M), not all detector will be bound to antigen, even at the highest

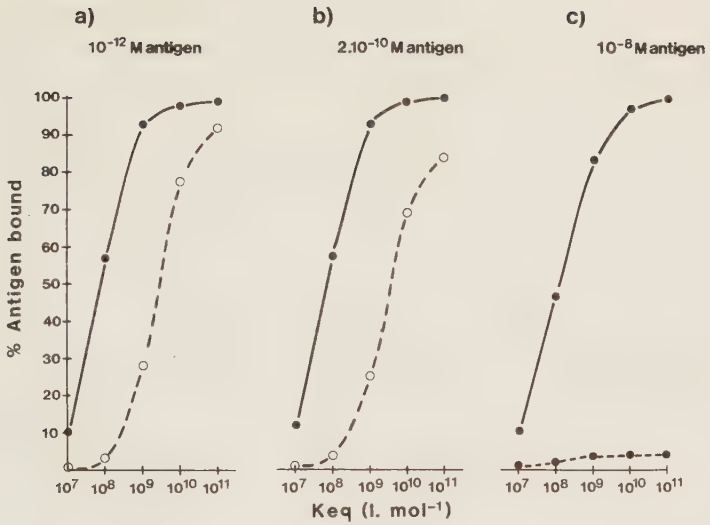

Fig. 19 **Formation of immune complexes in a homogeneous phase: computed curves of proportion of antigen bound.**

The proportion of antigen bound to antibody after 20 hours incubation (20 hr rather than equilibrium was taken because at low antibody concentrations at high K_{eq} values equilibrium was reached very slowly see R.7.2.1.4), has been computed using the algorithm described in M.7.4. Bivalent antibody and a 10^5 M^{-1} s^{-1} forward rate has been assumed. In each panel the algorithm has been solved for the range of values listed in table 19 (abscissa), and for two different antibody concentrations, 7 10^{-9} M = 1 µg/ml (solid line) and 2 10^{-10} M = 30 ng/ml (dashed line). The different panels show the effect of using the different antigen concentrations given below (the figures in brackets after the concentrations below indicate the % by mass of a 40 kD protein in a 800 ng protein mixture in 100 µl of solution which would correspond to these molarities): a) 10^{-12} M (0.0005%); b) 2 10^{-10} M (0.1%); 10^{-8} M (5%). The amount of antibody is limiting at the lower concentration of antibody used to compute the curve shown in panel c.

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K_{eq}. To bind large (>10%) amounts of detector molecules with low K_{eq} values (e.g. Protein A) requires a large molar excess of antigen (e.g. a 50 fold molar excess of IgG - as antigen) is required to obtain 50% binding of Protein A). The potential effect of K_{eq} on sensitivity is most clearly illustrated in

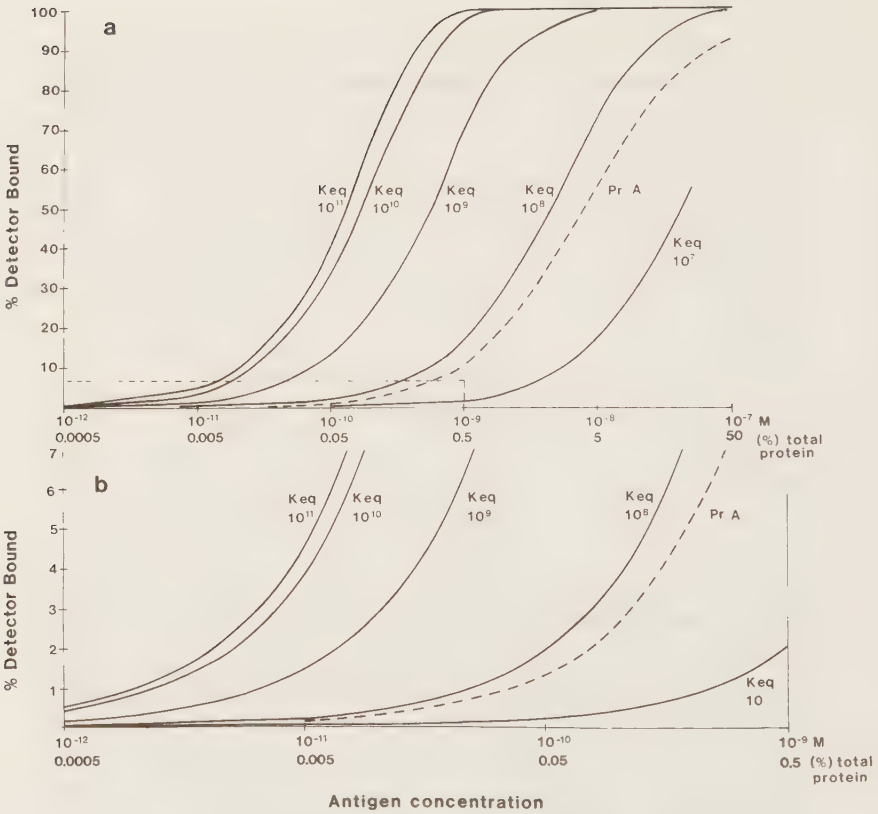

Fig. 20 **Effect of equilibrium constant on the binding of very dilute detector (anti-antibody or Protein A) to immune complexes in a homogeneous phase: computed curves of proportion of detector bound.**

a) proportion of detector (0-100%) bound to immune complexes after 20 hours (see legend to fig. 19) as a function of antigen concentration (abscissa, note log scale).

b) detail of panel a) indicated by the dashed box, showing detector binding at limiting antigen concentrations ($<10^{-9}$ M): the scale of the ordinate has been expanded 10 fold compared to panel a).

Bivalent IgG antibody at a concentration of $2\ 10^{-10}$ M (30 ng/ml) has been assumed, other computational parameters and details of computation are as detailed in the legend to fig. 19. The dashed line in both panels shows binding of Protein A at a concentration of $2.5\ 10^{-10}$ M (10 ng/ml) assuming a K_{eq} of $1.3\ 10^{8}$, forward rate of $10^{5}\ M^{-1}\ s^{-1}$ and monovalent binding.

fig. 20b in which the expected level of detector binding at low antigen concentrations is shown with an expanded vertical scale. If I assume that 0.2% binding (corresponding to about 500 cpm ^{125}I if the same amount and specific activity of detector were used as in a standard IRMA and if all the labelled detector were active - but see R.7.3.2.2) were to represent a lower limit to detection of detector, then examination of fig 20b reveals the following detection limits for antigen (table 20).

K_{eq}	Antigen Molarity	pg antigen of MW (kD) 20	40	160	Number of molecules
10^7	10^{-10}	200	400	1600	$6.0\ 10^9$
Protein A	$2\ 10^{-11}$	40	80	320	$1.2\ 10^9$
10^8	10^{-11}	20	40	160	$6.0\ 10^8$
10^9	$2\ 10^{-12}$	4	8	32	$1.2\ 10^8$
10^{10} } 10^{11} }	}approx. $2\ 10^{-13}$	0.4	0.8	3.2	$1.2\ 10^7$

Table 20 **Theoretical detection limits for indirect immunoassay.** Assumes 1:1:1 antigen:antibody:detector complexes. Antigen molarity indicates lowest concentration of antigen at which bound detector (0.2% binding) can still be detected. Number of molecules calculated on the basis of 0.1 ml reaction volume.

Assuming 11.3 pg protein/Drosophila cell nucleus (see R.9.1.1.3), 800ng nuclear protein is equivalent to the nuclei from $200\ 10^{-9}/11.3\ 10^{-12} = 8\ 10^4$ cells. This would mean that if both a monoclonal antibody and a detector molecule were to have site binding constants of 10^{11} that an antigen with a frequency of $1.2\ 10^7/8\ 10^4 = 150$ copies/nucleus would in theory be detectable using unfractionated nuclear protein.

I show in sections R.7.3 and R.8.3 that such a limit could be approached experimentally to within a factor of 10. These calculations are of value because, by applying binding

theory to solid phase assay systems, experimental factors limiting the performance of the assay can be located. Examples are presented in R.7.3.4, where the amount of sufficiently affine first antibody is probably limiting, and in R.7.4.5 where the amount of sufficiently affine second antibody is probably responsible. This allows possible improvements in the assay to be chosen on a logical basis. The analysis above suggests that, were it possible to obtain a sufficiently affine monoclonal/detector combination, an increase in assay sensitivity of only 60 fold (by increasing, say, specific activity of detector by a factor of 5 and by enriching a nuclear fraction a factor 12 with respect to the relative amount of an antigen) would allow the detection of an antigen with a frequency of only 10s of copies/diploid nucleus (see also R.9.3).

It must be stressed that for the purposes of simplicity, the theoretical calculations have been carried out assuming homogeneous phase conditions. It is however clear (R.7.4.4), that, to a first approximation, the equations used to describe a homogeneous phase reaction can also be used to describe the system properties of a solid phase assay. What is not clear is what the relationship is between the numerical value of a binding constant derived from homogeneous phase reaction and which describes a heterogeneous phase reaction (R.7.2.1.5).

R.7.2.1.4 Kinetics of complex formation

If estimates of k_{+1} or k_{-1} are available, the amount of complex at any time during the reaction can be calculated by integration of the expression:

$$\frac{dB}{dt} = (n[TAb]-[B])([TAg_d]-[B]) - k_{-1}[B] \qquad \text{Eq. (3)}$$

to which there is an analytic solution (see M.7.4).

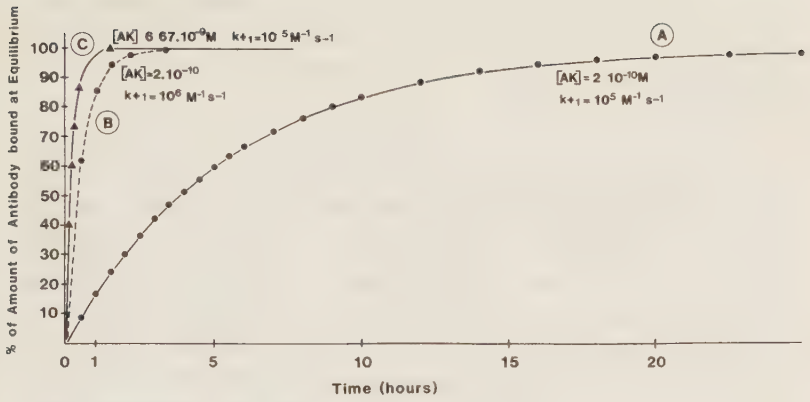

Fig. 21 **Time course of immune complex formation in homogeneous phase.**

The time course of reaction of an antibody with a K_{eq} of 10^{10} l mol^{-1} binding to antigen at a concentration of 10^{-11} M has been computed using the algorithm described in M.7.4. for the 3 different sets of conditions tabulated below:

	k_{+1} (M^{-1} s^{-1})	Antibody concentration (M)	(µg/ml)
Curve A	10^5	2 10^{-10}	0.03
Curve B	10^6	2 10^{-10}	0.03
Curve C	10^5	7 10^{-9}	1.0

The amount of antibody bound at equilibrium under the conditions of curves A and C would be 3.9% of the total IgG in the system (i.e. 7.8 10^{-12} M - 78% saturation of antigen); under the conditions of curve B 0.15% of the total IgG in the system would be bound (i.e. 9.9 10^{-12} M - 99% saturation of antigen).

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Computation of the time taken to reach equilibrium (99% of the equilibrium value), show that given a forward rate of 10^5 M^{-1} s^{-1} that in antibody excess equilibrium will be reached in times on the order of 1 hour or less (fig. 21, curve C). At low concentrations of antibody, however it may take several hours to reach equilibrium, even at high values

of K_{eq} (fig. 21 curve A: $t_{1/2}$= 4hr). The sensitivity of the time course to variation in k_{+1} may be judged by comparing curves A and B in fig. 21 which show the time course of a reaction of an antibodies with the same K_{eq} values, but with different k_{+1} values.

R.7.2.1.5 Relevance of homogeneous phase binding to heterogeneous phase assay

It is difficult to judge how relevant the binding constants observed in homogeneous phase reactions will be to solid phase binding.

It is possible to show that the path of a diffusing molecule causes it to be retained in the neighbourhood of a surface with which it has collided ("constrained path" effect, BERG and PURCELL 1977, see R.7.2.3.4). On the basis of this mechanism the theory of DELISI (1981) predicts that the equilibrium constant for antibodies binding to antigens forming part of the surface of a particle may be increased by a large factor (on the order of 10^4 for a 10 µ diameter particle), compared to the same reaction in homogeneous phase.

This theory is based (implicitly) on the assumption of freely diffusing, statistically distributed particles, with relatively small distances between them, although the mean of this distribution must presumably be much larger than the mean distance between the molecules on the surface of the particles. At any rate the spatial distribution of the particles is not explicitly considered. It may be a trivial exercise for a physicist to adapt the theory to a single "particle" with the dimensions of a microtitre plate well but, unfortunately, I have been unable to find any published work, at least in the immunological literature, in which this problem has been adressed. Nonetheless, in general, with increasing particle size, K_{eq} is expected to increase. If this theory were correct, it would mean that any antibody with a site binding constant greater than 10^6 l mol^{-1} in homogeneous

phase reactions would, effectively, become irreversibly bound to solid phase antigens. This would have a secondary effect: the magnitude of the "constrained path" effect predicted by DELISI would be so great, even for univalent ligands, that any further enhancement to binding caused by bi- or multivalent binding (see R.7.2.2.1) would not easily be detectable.

The DELISI theory, in its simplest form, does not seem to be correct, at least quantitatively (see below), perhaps because some of the assumptions about surface effects are oversimplified. For example, the correct expression for antigen and antibody activity must be found. This may not be simply the number of molecules in the system divided by its volume, a convention which I have adopted, for the sake of simplicity, in the rest of this work. It is probably necessary to take into account the concentration of the particles, as well as the reacting molecules at their surface, since this will determine the collisional frequency with a surface, which may be many orders of magnitude lower than for individual molecules (see R.7.2.3.3), and would presumably serve to counteract the "constrained path" effect to some extent. Assuming, however, that the "constrained path" effect is in principle correct (see R.7.2.3.4), equilibria reached in heterogeneous phase reactions may nonetheless differ from those reached in homogeneous phase, even if not to the extent predicted by DELISI.

The data of MASON and WILLIAMS (1980) comparing antibody binding to solubilised cell surface antigens and to intact cells (cell diam. 10 μ, area 10^{-7} cm^2) and the data of HOLMES and PARHAM (1983) on antigen binding to solid phase antibody (area approx. 1 cm^2) clearly rule out increases in K_{eq} as great as those predicted by the DELISI theory. Furthermore the valency of binding clearly was important in both the cited studies (see also R.7.2.2). However, the data is not sufficiently precise to rule out differences between homogeneous and heterogeneous phase binding equilibria on the order of a factor 10.

Independent of the equilibrium position which is finally reached it is clear that the rate at which equilibrium will be reached in solid phase assay will be slower than in homogenous phase (R.7.2.3.3) and the effect of washing will be reduced (R.7.2.3.4).

In summary it is conceivable that the sensitivity of solid phase assay could differ (favourably) from that of a hypothetical homogeneous phase assay. The quantitative extent of the putative advantage remains uncertain in the absence of adequate theory, but the very limited number of experimental observations available suggest that, except where the valency of binding is affected, it is small. If these observations can be generalised, they suggest that the equilibria computed for homogeneous phase reactions may provide a useful gross estimate for the sensitivity of solid phase assays.

R.7.2.2 Effect of heterogeneous phase (solid/liquid) reaction on antibody-antigen binding

Adsorbing one of the reactants in an immune reaction to a solid phase has two major effects:

1) the absorbed component is immobilised and forms some kind of lattice on the solid phase.

2) the collisional frequency between molecules of the two reacting species will be lower than in a homogeneous phase and will be dependent upon the geometry of the reaction vessel.

The first effect may have consequences upon the strength of antigen-antibody binding (R.7.2.2.1 - R.7.2.2.7) and the second (R.7.2.3) may affect the time course of the reaction.

R.7.2.2.1 Inactivation of adsorbed antigen

There are two principal mechanisms which might cause the inactivation of adsorbed antigen. Firstly, the degrees of diffusional and rotational freedom available to each adsorbed

molecule will be limited compared to those available in solution (DELISI 1981). However because the forward reaction of most protein-binding antibodies is not diffusion controlled, the constraint to reaction would have to be very great to become rate limiting. The rate of intermolecular collision would have to be lowered by a factor of about 10^3 to become rate limiting (see R.7.2.3). Secondly, a proportion of the absorbed antigen might no longer be able to react with antibody, either because its reactive sites were to become inaccessible as a result of absorption (steric effect - which might not be absolute) or because they might be destroyed (e.g. by denaturation as the result of surface forces involved in absorbtion). The effective concentration of the absorbed component would thereby be reduced.

To date, there is only one published study (KENNEL 1982) of the reactivity of solid phase bound antigen (using a single, pure antigen). This study showed a factor 10 reduction in the reactivity of antigen adsorbed to polystyrene and showed that the degree of inactivation was dependent on the nature of the surface to which the antigen was bound. Because pure antigen was used in this study, it is also possible that excluded volume effects (see R.7.2.2.2) might have played a role in limiting antigen reactivity. Whatever the mechanism, it is clear that the assumption that all antigen bound to a solid phase is available to antibody must be justified before any attempt is made to use antibody binding to provide absolute estimates of antigen amounts. Unfortunately, there is no way to estimate the magnitude of such effects a priori and they would have to be determined empirically. This would be an almost impossible undertaking, given an unknown antigen making up a very small fraction of an antigen mixture. Model studies, using known amounts of a number of different proteins in artifical mixtures might be sufficient to allow the range inactivation which occurs to be estimated.

R.7.2.2.2 Strength of antibody binding: increased "functional affinity" of bivalent antibody for solid phase antigen

In this section it is assumed, for the purpose of argument, that if an antibody is only capable of binding monovalently to an antigen bound to solid phase, the strength of binding should be the same as in homogeneous phase i.e. the extent of reaction will be determined by the site binding constant of the antibody and the concentration of each of the reactants. For simplicity, the concentration of the solid phase reactant is considered to be the number of molecules bound to the solid phase divided by the volume of the reaction mixture, despite the fact that this assumption may not justified (R.7.2.1.5, R.7.2.2.1, R.7.2.3.4).

If the antibody is able to bind to the adsorbed antigen with more than one valence, then the strength of its interaction with antigen may be drastically increased. The increased strength of binding of antibodies bivalently bound to surface antigens has been observed experimentally, using polyclonal antibodies, by GREENBURY et al. (1965), by HORNICK and KARUSH (1972) and, using monoclonal antibodies, by MASON and WILLIAMS (1980). More recently the effect has been studied in detail using bivalent monoclonal antibodies binding to bivalent antigens in solution (HOLMES and PARHAM 1983). The increase in the effective K_{eq} of the binding of antibody **molecules** (the site binding constant remains, by definition, unaffected) compared to monovalent binding appears to be by a factor lying between 10^2 and 10^4.

Arguments can be presented at a number of different levels to explain this phenomenon. One can consider the solid phase bound antigen as if it were a large polymeric molecule. Then, in contrast to the **inter**molecular reaction which is required for the binding of the first arm the antibody, the binding of the second arm can be regarded as an **intra**molecular rearrangement occurring with high probability.

Two factors are likely to control the exact level of probability. One is the frequency of the internal motion of

the IgG molecule which will allow the molecule to adopt a configuration which is energetically favorable for the binding of both arms (CROTHERS and METZGER 1972). The antibody molecule is known to be very flexible at the hinge region (VALENTINE and GREEN 1967, WRIGLEY et al., 1983) and an experimental determination of the frequency of motion of the two Fab arms relative to one another suggests motion with approximately Megahertz frequency (10^7 vibrations per second) with an amplitude of approximately 30^o, equivalent to a difference of about 20 Å in the distance between the tips of the Fab arms - range 60 Å to 80 Å (YGUERIABIDE et al., 1970). In addition to this motion, more recent studies indicate more complex motions, with even greater greater amplitudes, but lower frequencies (HANSON et al., 1981, REIDLER et al., 1982).

The other factor likely to influence the probability of reaction is the geometry of the antigen lattice. A model for the specific case of a regular one dimensional lattice has been presented by PINCUS and RENDELL (1981), and considers the effects of antibody concentration (in the presence of excess solid phase antigen), site binding constant, enhancement of second arm binding and of excluded volume on the fraction of antibody bound. Excluded volume is a measure of binding space unavailable to an antibody. Particular geometrical arrangements and steric exclusion by previously bound antibodies can both contribute to exclusion on a two dimensional lattice as the examples in fig. 22 show. The value of this parameter will determine how many neighbouring binding sites have an effect upon each other (i.e. are correlated) and this in turn will determine the proportion of antibody which is able to bind either mono- or bivalently as a function of lattice occupancy. The value of the excluded volume parameter will be dependent upon the packing density of antigenic determinants on the solid phase: LEHTONEN (1981) has shown this experimentally using haptens bound to a solid phase at different densities.

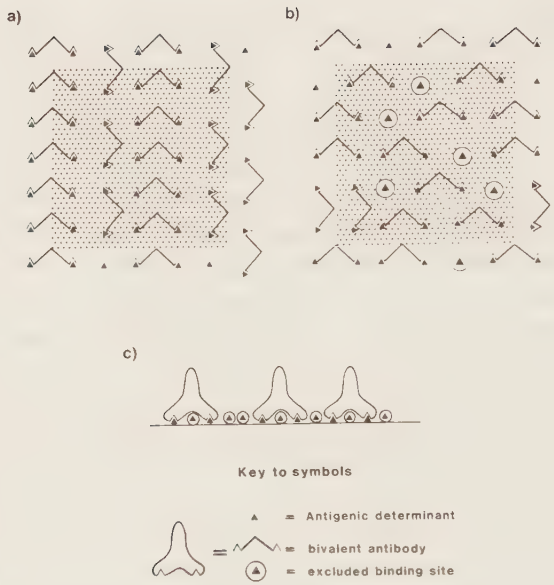

Fig. 22 **Theoretical examples of solid phase excluded volume effects.**

a) and b) geometrical effects: schematic representation of bivalent antibody binding to antigenic determinants arranged in a regular 5 x 5 lattice (shaded square). The spacing between antigenic determinants (triangles) is such that antibody is only able to bind horizontal or vertical, but not diagonal, neighbours bivalently. A 7 x 7 matrix has been drawn to show how all the antibody valences are satisfied, but in considering the proportion of **determinants** bound, the terminal elements in each row and column (unshaded) have been neglected because of edge effects (in practice in a 10^3 x 10^3 array edge effects would be negligible).

a) how it would theoretically be possible for all determinants in the lattice to be bound.

b) an arrangement of bound antibody which would result in exclusion of 4 (16%) of the antigenic determinants (circled) from bivalent binding because there are no free neighbouring determinants in the same row or column.

c) Steric effects. A differerent antigen lattice is supposed, with more closely spaced determinants than in a) and b). For clarity only a single row of the lattice is shown. Previously bound antibody molecules occupy the space which an incoming antibody molecule would have to occupy in order to bind. Therefore determinants which are neighbours to previously bound ones are rendered physically inaccessible. Geometrical and steric exclusion effects could occur additively.

The model of PINCUS and RENDELL (1981), (i.e. a one dimensional lattice) may appear simple. However, use of the descriptive algorith to provide quantitative estimates of antibody binding, even assuming a lattice in which only a single antigenic determinant on either side of a bound antibody arm is excluded, requires the solution of a polynomial expression with a quartic term and much additional computation. **For two dimensional lattices not even a computational solution is possible since no binding algorithm is currently available.**

Nonetheless one would intuitively expect two of the qualitative properties of the one dimensional lattice solution to apply, even if the quantitative details differ. These are that:

1) if excluded volume effects occur, then at all concentrations antibody may bind to antigen less than quantitatively, even though the antibody is very tightly bound.

2) biphasic binding curves as a function of antibody concentration would be expected if there was enhanced binding as a result of second arm attachment giving rise to an increase in the "functional affinity" of bivalently bound molecules. This is because, at low antibody concentrations, bivalent binding would predominate until almost all antigen binding sites became bound and a plateau in the binding curve would be observed. At very high antibody concentrations, however, monovalent antibody binding would predominate and the total amount of antibody bound would be greater, although more weakly bound.

In practice the density of antigen molecules bound to microtitre plates varied between $1.5\ 10^{12}$ and $5\ 10^{12}$ molecules cm^{-2}, see R.7.3.5). At the higher density, if the antigen molecules were arranged uniformly in a square array, the distance between molecules would be about 7 nm, approximately equal to the the distance between combining sites on the two arms of an bivalent antibody molecule. The

actual geometry of antigen arrangement is not known, but would presumably be dictated by the structure of the plastic polymer from which the plate was constructed, and both regular and random arrangements are imaginable. Any deviation from a regular square array would tend to cluster the molecules in at least one dimension, thus both bivalent binding and excluded volume effects appear to be possible events using microtitre plates as the solid phase support for IRMA and ELISA.

However, since IRMA and ELISA were usually performed using mixtures of proteins, of which the antigen often formed only a small fraction, to coat the solid phase, not every lattice site would be filled by an antigen molecule. Neighbouring pairs of lattice sites filled with antigen molecules would be still rarer, their frequency falling approximately as the square of their fractional abundance. However, given the large increases in functional affinity afforded by bivalent binding they might still be important. For example, consider a monoclonal antibody whose site binding constant is 10^7, but whose functional affinity (bivalent binding) is approximately 10^{10}. A glance at figure 19 shows that at any antigen concentration at most 10% of antigen would be bound monovalently by antibody at equilibrium. In contrast almost 100% of the antigen arranged such that it could be bound bivalently would be bound (neglecting any excluded volume effects). Given a true antigen frequency of 1%, 0.1% of the total antigen would be bound monovalently and approx. 0.01% bivalently. Thus, even though potential bivalent sites occurred with a frequency only 1% of the monovalent binding sites, antibody bound at the bivalent sites would account for more than 10% of the antibody bound. If the k_{-1} of the monovalently bound antibody were large, substantial losses of monovalently bound antibody during subsequent washes would be expected, whereas bivalently bound molecules would remain stably bound. As a result bivalently bound molecules would predominate in the population of bound antibody molecules finally seen by detector.

Given a high affinity detector molecule ($K_{eq}>10^9$), fig. 20 indicates that the signal from this small proportion of bivalently bound antibody would still be quite adequate for antigen detection. The amount of antibody bound would however give very misleading information about the amount of antigen generating the signal (the amount of antigen and the amount of bound antibody differ by two orders of magnitude). The above example is entirely hypothetical, but it does serve to emphasize the necessity for exercising caution in interpreting antibody binding data.

R.7.2.2.3 Tests for bivalent binding to solid phase antigen

If bivalent binding is important in generating the signal from a solid phase assay, then there are two kinds of test which might indicate how great a role this kind of binding might play. Firstly, if the affinity of binding by Fab fragments were markedly less than that of intact antibody this would indicate that bivalent antibody binding was substantial and that the site binding constant of the antibody was low. Secondly if there were asymmetry in binding behaviour, depending upon whether antibody or antigen was attached to the solid phase, this might also indicate bivalent binding. Whereas antigen attached to the solid phase would be expected to support bivalent binding, antibody bound to the solid phase could bind monovalent antigen independently to each of the solid phase bound arms, but no enhancement of binding by attachment of the second arm would be expected. Thus, using solid phase bound antibody, the extent of binding would be determined entirely by the site binding constant.

Using antigen at densities comparable ($3\ 10^{12}$ and $6\ 10^{12}$ molecules cm^{-2}) to those used in the model binding studies reported in this work, a large discrepancy was observed by LEHTONEN and EEROLA (1982) when they compared antibody affinities to a hapten estimated by equilibrium dialysis

binding and solid phase ELISA using hapten covalently attached to the surface. Binding by low affinity antibodies in ELISA was very much greater than that expected on the basis of the affinities determined by dialysis. These results might well reflect an assymmetry equivalent to the difference between attaching either antigen and or antibody to the solid phase (their ELISA results used solid phase antigen and the equilibrium dialysis results would be equivalent to attaching antibody to solid phase). Alternatively, surface binding effects (DELISI 1981, R.7.2.1.5 and R.7.2.3.4) might be invoked to explain these observations. An examination of the behaviour of Fab fragments in the ELISA system would have allowed these authors to suggest a mechanism for their results.

R.7.2.2.4 Special cases of bivalent binding. (i): bivalent antigen

There is one important exceptional case in which bivalent binding by antibody might occur independently of the proportion and geometry of solid phase antigen, and in which no asymmetry in the the strength of binding dependent upon which component was bound to the solid phase would be expected. This is when the antigen itself is bi- or multivalent. Two particular instances when this might occur are pertinent to this work, 1): particles which contain more than one copy of the same protein (possibly hnRNP particles ?) and 2): when the detector molecule is an antibody and directed against Fab determinants. Such a detector (Rabbit anti-mouse Fab - RAM-Fab) was used in IRMA, and it is certainly conceivable that at least a subpopulation of this monospecific but polyclonal antibody might only have bound mouse antibody significantly when it was able to bind both arms of the murine molecule (see R.7.4.5). Although, using such antigens, no asymmetry in binding affinity would be expected with respect

to the reactant bound to solid phase, Fab fragments would still bind to solid phase antigen very much less efficiently than intact molecules if their site binding constant were low.

R.7.2.2.5 Special cases of bivalent binding, (ii): antibody interactions

When two antibodies which bind to different determinants on the same antigen molecule, but with different affinities, another special case of bivalent binding occurs in which the more strongly binding antibody "enhances" the binding of the more weakly binding one. This effect has been reported for a number of different antigens, mainchains of HLA and H2 (HOLMES and PARHAM 1983, HOWARD et al., 1979, LEMKE and HÄMMERLING 1981), $ß_2$-microglobulin (LIABEUF et al., 1981), IgG (OI and HERZENBERG 1979, NEMAZEE and SATO 1982) and human chorionic gonadotrophin (EHRLICH et al. 1982). The mechanism by which such enhancement occurs is that the more strongly binding antibody binds two antigen molecules, effectively creating an antigen dimer, to which the antibody with the low site binding constant can then bind bivalently and with high functional affinity. Although this mechanism has only been stringently demonstrated in one particular case (HOLMES and PARHAM 1983), the existence of a significant proportion of Ab_2Ag_2 "limit complexes" when using polyclonal antibodies (AREND et al., 1972) and the variety of proteins for which enhancement has been observed (citations at the beginning this section) suggest that such interactions occur commonly in the immune system. This kind of bivalent antibody binding could not have occured using the assay procedures described in this work, but might be exploited in future in "sandwich assays" (solid phase-antibody-antigen-antibody*) to achieve reliable quantitation of particular antigens.

Apart from "limit complexes", network formation, (Ab_nAg_n polymers) leading to precipitation, can of course also occur

in a one antigen two antibody system in solution. Given the site binding constant of each antibody, the range of conditions under which Ab_2Ag_2 formation will predominate can be predicted theoretically (NICHOL and WINZOR 1976).

R.7.2.2.6 Special cases of bivalent binding. (iii): limited bivalent binding by high affinity antibody

Heterogeneous phase binding is also encountered when antibody binds to antigens which are mobile in the plane of a membrane. The 2-D lattice is in this case dynamic. Some of the theoretical aspects of mono- and bivalent membrane binding by antibody are discussed in TRUCCO and DePETRIS (1981), however experimental observations with monoclonal antibodies (MASON and WILLIAMS 1980) have revealed some supplementary phenomena which have not yet been adequately explained. Although these authors were able to observe both mono- and bivalent antibody binding they found -paradoxically- that, although able to bind bivalently, the most affine antibody (W3/25) bound predominantly monovalently. A polyclonal antibody has been described (DePETRIS and RAFF 1973, HEATH 1983) whose behaviour is analagous; it is functionally monovalent, although the antigen to which it is bound can be crosslinked (e.g. by addition of anti-antibody). Possibly the mechanism of high affinity binding is such that binding of a single arm poses steric constraints on the rest of the antibody molecule and hinders binding of the second arm. Alternatively simultaneous binding of both antibody arms to suitably arranged antigen pairs (i.e. a third order reaction mechanism) might play an important role in bivalent binding. In this case, the equilibrium level of bivalent binding would only be reached very slowly unless monovalently bound antibody were released sufficiently frequently to allow rare third order events to occur.

The observation that antibodies with high site binding constants show limited bivalent binding is at present

restricted to antibodies against membrane antigens. There is however no a priori reason why, if either of the two mechanisms I have suggested above is correct, such effects could not also occur when antigen molecules are attached to a rigid surface.

R.7.2.2.7 Caveat Emptor

To summarise: both a priori considerations and observed binding behaviour reveal that bi- and multi- valent antibody-antigen attachment can give rise to unpredictable effects on binding strengths and efficiencies. In addition antigen may be inactivated to an unknown extent by adsorption. These effects greatly complicate any analysis of antibody binding data. It seems reasonable to suggest that some estimate of each of these factors (site binding constant, extent of bivalent binding, antigen inactivation) should be acquired before any quantitative estimates of solid phase bound antigen, made on the basis of amounts of bound antibody, can be trusted.

R.7.2.3 Translational movement of molecules in liquid phase to the vessel walls: effect on reaction rate in solid phase assays

R.7.2.3.1 When is translational movement rate limiting? A comparison of dimensions in homogeneous and heterogenous phase reactions

Much of the discussion in the preceding paragraphs has been based upon the assumption that equilibrium has been reached. As fig. 21 shows, the time course of antigen-antibody binding in homogeneous phase can be rapid (taking minutes to hours to reach equilibrium), even with reactant components in the subnanomolar range. However, in homogeneous phase at 0.1 nM concentrations, the mean of the shortest distance between molecules is on the order of 10 microns, and the collisional

frequency at the start of the reaction will be on the order of 5-10 collisions molecule^{-1} second^{-1} (VON SMOLUCHOWSKI 1916) or about 10^{11} - 10^{12} collisions s^{-1} for the reaction volume (100 µl) usually used in a microtitre plate well for solid phase assay. When one of the reacting components is constrained to a solid phase and the solid phase comprises the walls of the vessel in which the reaction takes place (as opposed to a granular solid phase such as a bead or a cell surface), then the mean shortest distance between reactants will be determined by the geometry of the reaction vessel. For a microtitre plate well the distances between reactants will be very much greater (approx. 100x see below) than in homogeneous phase. For a given amount of solid phase reactant, this means that, unless the liquid phase reactant is present at a concentration at least 10 fold greater than that required to saturate binding of the same amount in homogeneous phase, then the rate of movement of molecules from the bulk of the liquid phase to the walls of the vessel would be expected to govern the rate of reaction.

Microtitre plates of different materials and from different manufacturers have slightly different geometries: regular cylinders, inverted truncated cones and inverted truncated cones superimposed on truncated cones. When such wells are filled with 100 µl of liquid (the amount assumed in all subsequent calculations) the mean radius of all the different kinds of wells in their liquid filled portion is 3.0 ± 0.1 mm and the depth of liquid in the wells is about 4mm (i.e. of similar dimensions to the radius). For the purposes of calculation a convenient approximation of such a vessel is a sphere of radius 2.9 mm. This body has a volume of 102 µl, but the area of vessel wall in contact with liquid in a sphere is 1.43x that of a microtitre well filled with 100 µl liquid.

On the basis of this approximation it is easy to calculate that 50% of the liquid phase molecules in the microtitre plate well will be located more than 0.6 mm away from its surface. If the components in the liquid phase are present in

10-fold excess of that required for saturation then the amount of reactant required to saturate the solid phase will be present in a 100 μ thick layer adjacent to the well wall. For reactions which are not intrinsically diffusion limited (anti-protein antibodies, see R.7.2.1.2), translational movement over distances of this magnitude will not appreciably slow the rate at which equilibrium is reached, compared to the rate expected in homogeneous phase.

R.7.2.3.2 Mechanisms involved in translational movement; diffusion, convection, stirring

Calculation of the mean time taken by molecules in the bulk phase to reach the vessel walls is complicated. A priori, translational movement of solute molecules from the liquid phase to the walls must be controlled by bulk motion of the liquid phase itself (thermal convection or the result of mechanical disturbance) and by movement of solute molecules within the bulk phase (diffusion). Factors which might affect bulk motion were uncontrolled variables in the immunoassays described in this work. Nonetheless, as the following argument should make clear, current knowledge of the physics of convection allows some estimate of the likelihood of its occurrence in microtitre plate wells.

In general, whether or not convection takes place in a liquid phase with a free surface (i.e. a liquid-air boundary) with a depth on the order of 1 cm and heated from below (cooled from above) is additively dependent upon surface tension effects (PEARSON 1958) and buoyancy effects (PALMER and BERG 1971). In an unbounded liquid layer of finite depth, but infinite extent, convection has been shown to occur when critical values of two supposedly dimensionless numbers, the Marangoni number, Ma, (for surface tension driven convection) and the Rayleigh number, R, (for buoyancy difference driven convection) are exceeeded (PALMER and BERG 1971). The numbers are defined as follows (PEARSON 1958):

$$Ma = \frac{-(d\sigma/dT)\, h^2\, \Delta T/dh}{\rho k v} \qquad \text{Eqn(4)}$$

where $d\sigma/dT$ = change in surface tension with temperature (approx. 0.15 dynes $cm^{-1}\ {}^{o}C^{-1}$ for water (NELKON and PARKER 1964)

ρ = specific gravity of water (0.998 at 20°C, 1.00 at °C)

h = depth of liquid (0.4 cm)

$\Delta T/dh$ = temperature drop across liquid layer ($^{o}C\ cm^{-1}$)

v = kinematic viscosity (1.005 10^{-2} Poise at 20°C, 1.78 10^{-2} Poise at 0°C)

k = thermal diffusivity, otherwise called thermal conductivity (PALMER and BERG 1971) or thermometric conductivity (PEARSON 1958) (1.43 10^{-3} cal s^{-1} cm^{-1} $^{o}C^{-1}$)

and

$$R = \frac{g\, a\, h^4\, \Delta T/dh}{kv} \qquad \text{Eqn (5)}$$

where g = acceleration due to gravity (981 cm s^{-2})

a = coefficient of thermal expansion (2.07 10^{-4} cm^3 cm^{-3} $^{o}C^{-1}$)

h, v, k and $\Delta T/dh$ are as defined for equation (4)

where not otherwise stated numerical values taken from BERG et al. (1966).

The critical values of Ma and R for pure water are 80 and 1800 respectively (PEARSON 1958). The temperature drop across an aqueous layer of depth approximately 1 cm allowed to evaporate freely at room temperature is about 1°C cm^{-1} (BERG et al., 1966; MICHEAU et al., 1983). Substitution of the values listed above gives the following critical depths for convection (table 23):

	Temperature of Liquid	
	0°C	20°C
Convection by surface tension	1.2 mm	0.87 mm
Convection by bouyancy	6.90mm	5.9 mm

Table 23 **Critical depth of liquid required for convection to be driven by a 1°C cm^{-1} temperature gradient**

The 20°C values compare with values of 0.8 mm and 4.7 mm calculated by MICHEAU et al. (1983), for surface tension and buoyancy driven convection respectively. i.e. there is good agreement about the critical depth for surface tension driven convection, but a difference of 1.2 mm in the estimated critical depth for buoyancy driven convection. Comparison of equations 4 and 5 above shows that this discrepancy can only have come about if MICHEAU et al. (1983) used different values for either g or a in eq. (5). It is unlikely that they would have used a different value for the gravitational constant, but if they used a value of $5.1\ 10^{-4}$ for the thermal expansion coefficient this would account for the discrepancy. Unfortunately, these authors give no details of their calculations.

For most pure liquids theory and observation have been shown to be in good agreement (PALMER and BERG 1971; BERG et al. 1966). However, for pure water, BERG et al. (1966) were unable to observe any convection in a vessel of 10 cm diameter with an open upper surface if it was filled to a depth of less than 1 cm, even when a stream of nitrogen (2 L min^{-1} over an area of 100 cm^2) was blown over the surface. They cite similar observations by other investigators. In contrast it is clear from the results reported by MICHEAU et al.(1983) that they were able to obtain convection in evaporating layers of salt solutions as thin as 1 mm. Unfortunately they do not give any details of the exact composition of the solutions they used. The reason for the discrepancy between the observations of BERG et al., and MICHEAU et al., with regard to the convective behaviour of aqueous solutions is unclear. The methods used by both sets of investigators were the same, as were the dimensions of the vessels they used. BERG et al., attribute the lack of convection in their "thin" aqueous layers to the presence of surfactant contaminants in their measuring apparatus. They suppose that this reduced the surface tension of the water to such an extent that surface tension driven

convection was eliminated. On the other hand, the presence of solutes in a solution may also contribute to convective instabilities; phenomena which are extremely complex and poorly understood (MOORE et al., 1983).

Convection will only occur if an temperature gradient can be maintained across a liquid layer. In the absence of deliberate heating, the only likely mechanism able to generate the necessary energy flux is the cooling resulting from evaporation. Both BERG et al., (1966) and MICHEAU et al., (1983) observed that the simple measure of placing a lid on the vessels they were observing completely prevented convection.

Taking theoretical expectations and experimental observations together (despite the discrepancies mentioned above) the only reasonable prediction which can be made about convection in microtitre plate wells under normal solid phase assay conditions is that its occurrence is highly unlikely. The reasoning can be subdivided into three sections: lack of a driving energy source, low surface tension of the aqueous solutions and dimensional effects.

The primary reason why convection is unlikely is that the microtitre plates are covered with lids. Under these conditions it almost certain that except for a very short period after sealing the plates that no net evaporation is taking place in the space above individual microtitre wells. If the lid fits poorly, evaporation may be taking place in the outermost wells on the plate, as the "edge effect" results indicate (R.7.3.1.2.5), but even under these conditions the results obtained from all except the outermost row and column of wells are uniform and identical with those obtained when the plates are fully sealed. Thus, provided that the plates and the solutions put in them are initially at temperature equilibrium, it is difficult to see from where the energy necessary to drive convection could come.

Even if a temperature difference between the top and bottom of a well, equivalent to that resulting from evaporation from an open vessel, were to occur (e.g. because of stratified air currents in the laboratory), it is still doubtful whether convection would be able to take place at most stages of solid phase assay. Apart from when very dilute protein solutions are used to coat the well surface with antigen, almost all other stages in the assay use solutions containing high concentrations of protein (1 mg/ml BSA) and/or detergent (Triton or Tween). Both protein and detergent are surfactant and would be expected to lower the value of $d\sigma/dT$ in eq. (4) such that surface tension driven convection would no longer operate. Buoyancy driven convection would also be unable to operate under solid phase assay conditions because the depth of liquid in the wells (4 mm) does not exceed the necessary critical depth for this mechanism to operate (independent of whether my estimate, table 23 or MICHEAU et al.'s estimate is used).

A further factor which is likely to hinder convection in microtiter plate wells is the small lateral dimension of the well itself. MICHEAU et al., (1983) have shown empirically that the wavelength of convective rolls is about twice the depth of liquid in which the convection is taking place. A minimum diameter of 8 mm would therefore be required for "normal" convection to take place. The diameter of the wells is however only 6 mm. No investigators appear yet to have investigated what occurs under such conditions. It is known that the effects of solid/liquid interfaces on convection are extremely complex and non-linear (ROSENBLAT et al., 1982), so it is hazardous to predict what might happen. However, intuitively, I would suspect more energy to be required to establish convective flow because of friction between convective flow streams and the vessel walls. Thus I would expect convection in small diameter vessels to be inhibited.

The above discussion assumes initial temperature equilib-

rium. Of course, if this condition is not observed e.g. a 0°C solution is pipetted into a plate standing at room temperature, it is clear that temperature gradients large enough to generate convective flow by either mechanism might occur. In one experiment, in which the time course of a binding reaction was being measured, and such a situation occurred by accident the results were uninterpretable.

It is worth noting in this context that plates incubated on ice in a cold room are exposed to temperature gradients which, because of the anomalous density change of water below 4 °C, could, in principle, give rise to buoyancy driven convective flow, although for the reasons discussed above it is thought to be highly unlikely that such flow would have occurred.

In summary, it appears that convection can be neglected as a major mechanism for translational movement. Might stirring, mechanical disturbance, be important? Uncontrolled energy inputs would be expected to give rise to non-uniform effects. However, experimental binding results (see R.7.3.3), were usually reproducible both from well to well and from plate to plate, both in the same experiment and from experiment to experiment. Thus, convection and mechanical disturbance must usually have had an influence which was either constant, despite the uncontrolled conditions, or so small as to be negligible. Finally, the good agreement between the observed time course of binding of antigen to the well walls (R.7.3.3) and that predicted by a pure diffusional mechanism (R.7.2.3.3) also argues for the absence of such effects.

R.7.2.3.3 Time course of diffusional transport

The effect of pure diffusion, in contrast to convection,

is both calculable and observable, although the calculation is complicated because the contribution of molecules from different regions of the liquid phase to the flux at the vessel wall is a probabilistic function of both time and distance which cannot be linearly transformed. Nonetheless, on the assumption that all molecules reaching the vessel walls are removed from solution, which is certainly justifiable for the case of limiting amounts of antigen binding directly to the solid phase, and possibly also for antibody binding reactions (R.7.2.3.4), algorithms are available (see M.7.5) which allows the amount reaching the surface of either spherical and cylindrical vessels to be calculated.

Fig 23a shows the time course of pure diffusional transport from the liquid phase to the walls for a representative protein (IgG) in a vessel containing the same volume as that usually used in a microtitre plate well. Fig. 23b shows the proportion of protein in the vessel which would be expected to reach the vessel walls as a function of vessel size. Curves a-d in each panel show the sensitivity of these functions to vessel geometry and to temperature (which affects the diffusion constant).

The time course of transport follows second order kinetics, as might be expected from Fick's second Law of Diffusion, with a half time of $1/k[A]$ where $[A]$ is the concentration of the diffusing species A at the beginning of each half time,

$$\text{i.e. } [A]/2 = 1/k\,[A]$$

and k is a constant determined by the vessel geometry and the diffusion constant of A. It is not immediately apparent from the algorithm used for calculation that the kinetics are second order. For comparison, curve e in fig. 23a shows the time course of a process with first order kinetics and the same half time as the diffusional transport shown in curve a. Note that, until half the initial concentration is reached, the rate of diffusive transport is more rapid than a first order process but that the rate drops off rapidly thereafter.

It is noteworthy that the proportion of solute which has reached the the vessel walls after 16 hours (an overnight incubation) is quite efficient (70% to 95%) (fig. 23b) regardless of whether a spherical or cylindrical geometry is assumed. However reactions can never be entirely quantitative. Even doubling the incubation period could only lead to

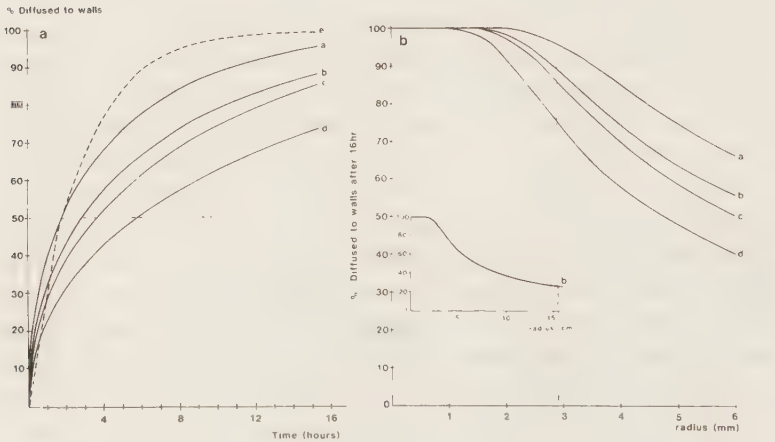

Fig. 23 **Characteristics of diffusional transport in small vessels.**
a) Computed time course of IgG to the walls of vessels with different geometries and at different temperatures.
The curves were computed as detailed in M.7.5, using the following parameters: radius of vessel (sphere or cylinder) 2.9 mm. Diffusion coefficient (20°C) 4.0 10^7 cm^2 s^{-1}, (4°C) 2.5 10^7 cm^2 s^{-1} (cf. M.7.5). The cylinder is assumed to have perfectly non-binding end walls, so its length is immaterial to the calculation, however a cylinder of height 3.8 mm would have a volume of 100 µl. Curve a) spherical geometry, 20 °C, b) spherical geometry, 4 °C, c) cylindrical geometry, 20 °C, d) cylindrical geometry, 4 °C, e) first order rate process with same $t_{1/2}$ as curve a).
b) Sensitivity of diffusive transport to vessel dimensions.
The proportion of IgG diffusing to the walls of spherical or cylindrical vessels during an overnight (16hr.) incubation has been computed as a function of vessel radius. Computional details and designation of curves as for panel a. Insert, curve b plotted for large vessel radii. Dotted vertical lines indicates radius of a microtitre plate well.

relative increases in efficiency on the order of 10%. The true geometry of a microtitre plates lies between that of a sphere and a cylinder. Obviously, when the amount of protein to be adsorbed is less than or close to the adsorbtive capacity of the well (see R.7.3.5), overnight incubations offer a practicable compromise between the speed and the completeness with which assays can be carried out in vessels of microtitre plate well dimensions. By contrast, if a vessel of double the radius were used, transfer would be less than 50% efficient even after an overnight incubation.

In considering the the rate of reaction of antibody with antigen it useful to be aware of the absolute rate of transport. The initial flux of molecules reaching the walls of the vessel, assuming an initial 2 10^{-10} M (0.2 nM, cf. fig. 20) concentration of liquid phase solute, would be 10^{7} molecules s^{-1}, or about 4 orders of magnitude less than the rate of encounter between two reactants both at this concentration in homogeneous solution.

R.7.2.3.4 Rates of reaction at surfaces

The curves shown in fig. 23 were calculated on the assumption that all molecules reaching the surface would become bound. This might be true for the process of adsorption, but it could not be true for antibodies with low binding constants if the equilibrium reached by the antibody-antigen reaction were the same as between equivalent amounts of reactants in homogeneous phase. Nonetheless, it is possible to show that almost every collision of an antibody molecule with the vessel wall will lead to an encounter with an antigen molecule. This is because the (random walk) path which a molecule follows once it has encountered a surface is likely to cause it to recollide with surface many times before escapes to bulk solution (> 2 molecular diameters from the surface - DELISI 1981). The exact probability P_{esc} that

antibody will **not** encounter an antigen molecule before escaping from the neighbourhood of the surface has been shown (BERG and PURCELL 1977) to be given by the relationship:

$$P_{esc} = \frac{\pi a}{(\pi a + Nr)} \qquad \text{Eq. (6)}$$

where a = radius of solid phase particle whose surface is being considered (spherical geometry assumed)
π = the constant π (3.14....)
N = total number of antigen molecules on the surface
r = radius of the antigen molecule

For a "particle" with the dimensions of a microtitre plate well ($a = 3\ 10^{-1}$ cm) and assuming $r = 3$ nm (30 $\overset{o}{A}$), P_{esc} will be small (<1%) for all values of $N > 5\ 10^{8}$. This value of N corresponds to about 0.05% occupancy of the surface area of a microtitre plate by a protein. Thus at all concentrations of antigen which one might expect to detect (given an equilibrium position similar to that found in solid phase, see fig. 20), >99% of the collisions of an antibody molecule with the vessel wall would be expected to lead to at least one encounter with an antigen molecule.

Even in homogeneous solution, with only the viscous and frictional properties of the solvent imposing constraints on rotational and diffusional movement, and assuming the highest forward rates possible (table 19, charge effects notwithstanding), 1000 encounters, on average, will be required before successful binding occurs. In considering solid phase binding, the probability P_n that a molecule will **not** have escaped from the surface neighbourhood before n encounters with antigen have been made will be given by:

$$P_n = (1 - P_{esc})^n \qquad \text{Eq. (7)}$$

It follows that, for all reactions where $1 - P_{esc} > 0.994$, P_{1000} will be greater than 10^{-3}. i.e. if the same number of encounters is required to give rise to successful binding on solid phase as in solution, the chances that the antibody in the solid phase assay will have reacted as a consequence of a

single collision with the vessel walls will be greater than if 1000 encounters had taken place in free solution, but only by a small margin and only assuming that the intrinsic site constant will not have been altered by solid phase binding (see R.7.2.2.1). P_{1000} obviously increases as a function of N (Eq. 6), and for values of $N > 10^{10}$ (about 1% of the well surface covered by antigen, $1 - P_{esc} = 0.9995$), approximately every second collision with the well wall would be expected to lead to successful binding. Thus at moderate antigen levels (1% of a mixture of proteins), a high proportion of collisions with the vessel wall would be expected to give rise to immune complex formation, and the reaction would proceed at very close to the rate of delivery of molecules to the vessel walls discussed in the previous section (R.7.2.3.3). This conclusion is critically dependent upon the assumption that the intrinsic site constant for reaction in solution and for a solid phase reaction are very nearly the same. As I showed in the previous section (R.7.2.3.3) the initial forward rate of a solid phase reaction in a vessel of microtitre well dimensions must be lower (by a factor 10^4) than that of a homogeneous phase reaction involving equal amounts of the two reactants in the same volume. The effect of this on the equilibrium position would be counteracted to some extent by the reduction in the reverse reaction rate (newly released molecules would have a greater chance of rebinding than in solution). As a result, the equilibrium position of the whole reaction might not, in the end, be greatly different from that found in solution.

An exact solution to the rate equation for solid phase binding is likely to be very complicated, being similar in form to that for sequential second order reactions, for which no analytical solution exists. As CANTOR and SCHIMMEL (1980) express it:

"Even for the simple bimolecular reaction the solution to the rate equation is complex. .....there is no convenient

analytical solution that is valid for general boundary conditions. .....It not suprising, therefore, that severe difficulties are encountered with the simplest [multistep] mechanisms."

One would intuitively suspect a zone of antibody depletion adjacent to the vessel walls, (i.e. a gradient) but because of back reaction effects (see below) the exact concentration profile of antibody would be difficult to predict. However, even if every collision were successful, the rate of diffusion from the bulk of the liquid phase to the walls would be expected to sets an **upper** limit to the rate and extent of reaction.

Not only will the time course of the reaction with which binding of antibody from an antibody solution to solid phase is established be affected, but also the rate at which a new equilibrium will be reached after washing. I can only describe the expected affects qualitatively. Initially, after washing, the concentration of newly dissociated material in the solvent layer adjacent to the vessel walls would be higher than that which would be expected for identical amounts in homogeneous solution or after extensive diffusive transfer had taken place. For instance, given washes with a mean duration of 5 min, in the absence of mechanical and convective influences and considering the fate of only those molecules which have escaped from the neighbourhood of the vessel wall, released antibody would only be present in significant quantities in a 300 µm layer adjacent to the well surface. This distance is twice the root mean displacement, given by the relation:

$$\sqrt{(\text{displacement})^2} = \sqrt{2\ D\ t} \qquad \text{(EINSTEIN 1905)}$$

where D=diffusion constant and t=time

This zone has a volume only 28% of that potentially available

in the vessel. The concentration of released antibody would therefore be 4 fold greater than if the wash were carried out with both reactants in a homogeneous aqueous phase. Rebinding would therefore occur at higher rate and the approach to equilibrium, compared to that in homogeneous phase, would be slowed. This effect is additional to the "constrained path" effect discussed in the first part of this section and would be dependent upon vessel geometry. Both effects would be expected to facilitate the detection of weakly binding antibodies.

A cautionary remark is necessary to close this chapter. The treatment of diffusive effects which I have given is very simplified. Electric charge, which is known to effect both reaction and diffusion rates (EIGEN and HAMMES 1963, CADMAN et al., 1981), has been neglected. I would however argue that the immunoassay conditions usually used (very dilute solutions, 140 mM NaCl) preclude any drastic influence from this source.

R.7.2.4 Complications imposed by using polyclonal antibodies: Antibody heterogeneity

Several types of polyclonal antibody were used in this study: the anti-H1 antibodies described in R.4.1, Rabbit anti-mouse Fab (RAM-Fab - produced by our group in Tübingen, RISAU et al., 1981), as the IRMA mouse monoclonal detection reagent, and a variety of commercial anti-antibody preparations - directed against various parts of the mouse or rabbit IgG molecule - for ELISA measurements. All these antibodies were affinity purified and therefore monospecific in the sense that the macromolecule against which they were directed was defined. Nonetheless, they were polyclonal, which means that they consisted of a heterogeneous collection of antibodies with different properties.

There are at least two basic kinds of variation which might be expected to occur within the population of molecules

making up a monospecific antibody against a protein antigen:

1) antibodies directed against different antigenic determinants on the same molecule (SACHS et al., 1972, ATASSI 1975, 1978, WAGENER et al., 1983b);

2) antibodies of different affinities directed against the same antigenic determinant (NISONOFF and PRESSMAN 1958, OWEN et al., 1982, LEHTONEN and EEROLA 1982, WAGENER et al., 1983b);

Two further kinds of variation might be expected as secondary effects of this primary variation:

3) differences in the concentration and/or mean affinity of subpopulations directed against different antigenic determinants (see e.g. WAGENER et al., 1983b, might give rise to different kinds of binding behaviour at different antibody concentrations);

4) in cases where the antigens used to produce the antibodies were themselves heterogeneous (e.g. IgG, see R.7.2.5), antibodies against antigenic determinants only found on subpopulations of antigen molecules.

Of course any or all of these variables might differ considerably between different populations of monospecific antibodies (e.g. from different rabbits/commercial firms).

Until the advent of hybridoma technology, the term antibody usually referred to a collection of molecules (see R.7.2.3). In the case of collections homogeneous with respect to determinant specificity (e.g. anti-hapten antibody), but heterogenous with respect to affinity, a simple test for extensive heterogeneity is given by using the graphical method based on Eq. (2) outlined in R.7.2.1.1: departures from linearity indicating major heterogeneity. A quantitative measure of heterogeneity can be obtained by estimating the standard deviation of the distribution of antibody affinities (PINCKARD 1978, PARKER 1976), on the assumption that the frequency of antibodies with different affinities within a population follows a log normal distribution or one similar to

it (e.g Sips function), which is symmetrically and continuously distributed about some mean affinity. Although the use of the Sips distribution has many intrinsic shortcomings (see FAZEKAS deSt.GROTH 1979), it is most widely used and can produce interpretable results (PINCKARD 1978). However in the few cases in which heterogeneity has been scrutinised in any detail, distributions of affinity have been found to be discrete (i.e. the antibody is oligoclonal) and highly skewed (FAZEKAS deSt. GROTH 1967, WERBLIN & SISKIND 1972, KIM et al., 1974, OTTERNESS and KARUSH 1982).

Neither of the above methods of testing for heterogeneity would be applicable to multivalent antibody-antigen interactions, because of the non-linearities introduced into the binding equations by the multivalence itself (CALVERT et al., 1979; NICHOL and WINZOR 1976). Thus to date there is no way to estimate the heterogeneity of most anti-protein antibodies from binding data alone.

In the following sections, where no information to the contrary is available, monospecific antibody populations are usually treated as if all their component antibodies interact with their targets in an identical fashion. It is important to bear in mind that this is only a convenient simplification. In some contexts (cf. R.7.3.4.2, R.8.2) it is obvious that such approximations are inappropriate.

R.7.2.5 Complications imposed by antigen heterogeneity

Each of the model antigens used in the next section is heterogeneous, but the quantitative extent of this heterogeneity is very different.

The protein fraction designated "histone 1" has been found to be a mixture of closely related protein variants, in all species in which it has been carefully investigated (COLE

1977, HOHMANN 1978). The variation which is found stems in part from differences in primary sequence (KINCADE and COLE 1966b, COLE 1977) and in part from different degrees of derivatisation with phosphate (HOHMANN et al., 1976) and/or ADP-ribose (MANDEL et al., 1982). It is certainly possible to produce antibodies which descriminate between different sequence variants (BUSTIN and STOLLAR 1973a,b, SLUYSER and BUSTIN 1974). Therefore, even though the protein used is pure in the sense that it only gives a double band following SDS-PAGE analysis, there is no guarantee that all molecules in the antigen population are equally capable of being recognised by antibody. Indeed, such effects might be expected from the 405/6 rabbit antibody preparation which was specifically raised against H1 subfraction 3 (cf. R.4.1). Such effects would compromise any attempt to accurately measure the binding capacity of the solid phase for antigen when this is estimated by means of antibody binding. In general, such effects would be expected to lead to underestimates of the amount of antigen bound.

The heterogeneity found in the histone 1 fraction is minor compared to that expected for IgG derived from whole mouse serum. Subclass, light chain type, variable region framework class variants and idiotype all possess antigenic determinants which can be discriminated by antibodies (FAHEY et al., 1964, GREY et al., 1971, MAGE et al., 1973, BONA 1981, RAJEWSKY and TAKEMORI 1983, ESHHAR et al., 1983). I show in R.8.2 and R.8.3 that this can impose limits on attempts at quantitative measurement using either IRMA or ELISA.

R.7.3 Model studies of solid phase assays

R.7.3.1 The range and limits of signal detectability

R.7.3.1.1 In IRMA

I have already described in the introduction to this

chapter (p.193) how an upper limit to amounts which can be measured is set by safety considerations. The question of lower limits is treated here.

R.7.3.1.1.1 Environmental background

In any counting technique sensitivity will be determined by the smallest difference that can reliably be discerned between counts resulting from a particular treatment (signal) and counts arising independently (background or noise). The threshold of detectability will be set by the amount of fluctuation between independent samples in each source of counts.

Neglecting for a moment any background arising from experimental procedures, there is one source of background which it is not possible to reduce using commercially available gamma counters and that is the environmental background. Energetic particles arising from the earth itself, from radioactive gas in the atmosphere and from cosmic rays probably all contribute to this background and it is not feasible to build a perfectly shielded counter for general laboratory use. Assuming that environmental radiation remains more or less constant, fluctuations in background counts will then be determined simply by counting statistics whereby the standard deviation (S.D) of the number of counts recorded will be given by:

$$S.D = \sqrt{\text{absolute nr. counts}}$$

This will clearly be dependent on the duration of counting. If fluctuations in the treatment counts are also determined mainly by counting statistics then, assuming a counting time of 1 minute and 36 cpm environmental background in Tübingen, a signal of approx 10 cpm (i.e. 46 cpm measured) can be calculated on statistical grounds (M.7.2) to set the lower

limit of detectability. This corresponds to about 10^6 molecules of a protein labelled with a stoichiometry of 1 mole ^{125}I/ mole protein. A similar limit (10 attomoles of ^{125}I) has been calculated by HUNTER (1978). Counting for longer periods would increase the sensitivity but only by a factor of about 3 for every factor 10 increase in counting time. Thus, under practical laboratory conditions, 10^5 - 10^6 molecules sets a theoretical lower limit to the number of molecules which could be determined in an IRMA assay using the ^{125}I isotope.

R.7.3.1.1.2 Non-specific binding background

In many of the model experiments (see e.g. R.7.3.4.1) it was possible to reduce background counts to near the theoretical minimum. In some experiments however, especially those using sonicated Kc nuclei as antigen, unspecific binding either of the monoclonal (or monospecific) antibody to BSA or of the second antibody (or protein A) to antigen was much greater (100 - 500 cpm). Furthermore, when several identical blanks were counted, the S.D. of the mean blank value was greater than that expected as a result of counting error: for means below 50 c.p.m. (above environmental background), an S.D. equal to the mean was not uncommon, but blanks greater than 100 c.p.m. had S.D.s which were approximately proportional to and ranged between about 10 - 20% of the mean. The S.D. of treatment means was usually somewhat less, ranging from 5 - 10% of the mean. When only duplicate measurements are made this extent of variability can be calculated to set a lower limit of signal detectability in the order of the range of the non-specific background values themselves i.e. 10^7 - $5.0\ 10^7$ molecules (assuming that a 5% error rate is acceptable). This result stresses the importance of trying to find conditions which minimise unspecific binding in IRMA assays whilst not affecting specific binding. An example of how this can be achieved is shown in fig. 24, which is taken

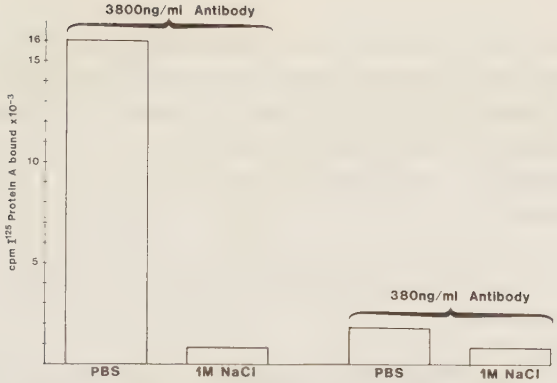

Fig 24 **Suppression of non-specific antibody binding by salt.** Microtitre plates were coated with BSA (0.5% w/v - 15 min) and incubated with the indicated amounts of monospecific anti-H1 antibody (rabbit 402) diluted either in PBS (140 mM NaCl) or in PBS (1 M NaCl). The different salt concentrations were also used in subsequent steps. The plates were washed, incubated with I^{125} Protein A (2.5 10^5 cpm), washed twice, drained, the wells separated and counted. Buffers used for the protein A incubation and subsequent steps contained 0.1% Triton X-100. Results shown are means of quadruplicate treatments.

from some early IRMA experiments and shows how it is possible to reduce unspecific binding of an anti-H1 antibody to serum albumin from 16,000 cpm to less than 1000 cpm by the simple expedient of adjusting the wash buffers used after the monospecific antibody binding step to 1 M NaCl. Notice that the binding of antibody to serum albumin is dependent upon antibody concentration, indicating a weak interaction between antibody and albumin (not necessarily immunological). This effect is found to different extents with different antibodies (cf. R.7.3.1.2.3). The binding of detector to different antigens was also variable.

Because of this variability, it is not possible to give a universal recipe for reducing background to a minimum: the exact conditions which gave the best results varied considerably from one antibody-antigen combination to another (see materials and methods for details). In general, protein in all the wash buffers and 0.1% Triton X-100 in the buffer

used after the radioactive reagent was bound sufficed to produce acceptable backgrounds. It should however be noted that when sera are used as a source of protein for minimising background that they should screened first because there can be large differences between different sera as table 24 illustrates.

	TREATMENT NUMBER					
	1	2	3	4	5	6
Treatment	Medium alone	Medium+ 10% FCS (Batch1)	Medium+ 10% FCS (Batch2)	Medium+ 40% FCS (Batch2)	Medium+ 10% HS (Batch1)	Medium+ 10% HS (Batch2)
non-specific binding to Kc nuclei (cpm)	74	290	323	540	1852	1937
(95% confidence limits)	(59-89)	(266-314)	(286-360)	(485-595)	(1822-1882)	(1856-2018)
attributable to serum component - Kc interaction (%)	-	65	66	85	89	88
% max. signal (= approx. 25% input cpm)	0.35	1.4	1.6	2.6	9.0	9.4

Table 24 **Serum components can be major source of non-significant binding.**

Microtitre plate wells coated with antigen (10 µg sonicated Kc cell nuclei input/ well) were incubated as indicated with media containing sera either in different amounts, from different species (FCS = Fetal Calf Serum, HS = Horse Serum) or from different batches from the same species. The wells were washed and incubated with ^{125}I RAM-Fab (85682 cpm, S.D.±1575, n=4) and then processed as in the normal manner for IRMA. Means and confidence limits calculated from quintuplicate values. The values in line 3 have been calculated after correction for binding of serum components to BSA-coated wells (not shown), and for binding of detector directly to Kc material (line 1, treatment 1).

Comparison of the result for treatment 1 in table 24 with those for treatments 2 - 5 shows clearly that binding of detector to serum components bound to Kc cell nuclei can be

significant. Assuming that a signal at least double background would be required for signal detection, even using the least reactive serum, antigen detectability would be limited to wells giving a gross signal of at least 500 cpm (approx. 250 cpm netto attributable to the specific antibody-antigen interaction). Comparison of columns 3 and 4 shows that non-specific binding is dependent upon serum concentration, although not in proportional fashion, suggesting some sort of saturable binding site. The source of serum can also be important, as the high (10% of the maximum binding possible) backgrounds produced by two batches of horse serum show. Not all batches of horse serum produce such high backgrounds as in this experiment: another batch of HS, which was used routinely, gave backgrounds no higher than those produced by FCS (results not shown).

These observations underline the conclusion that it is dangerous to make facile generalisations about the properties of such complex and variable mixtures of proteins as sera. If they must be used in sensitive immunological procedures, their characteristics should first be empirically tested.

R.7.3.1.1.3 Sources of variation: advantages of replicate determinations

The variance of both background and treatment values greatly exceeded that attributable to counting errors. This indicates that other sources of error must occur in the assay and means that little or no advantage is to be had from increasing the duration of counting. There are many possible sources of the observed variability; small errors in pipetting, the method does involve 7 - 10 pipetting steps, small gradients in temperature across the plates or non-uniform binding of protein at different positions on the plate etc.. However, given this degree of variability, it should be noted that a considerable increase in sensitivity may be obtained by using adequate replication (triplicate or quadruplicate values

of the same treatment, each obtained from a separate well). As an example, assuming an S.D. of 20% of the mean, it is easy to calculate (see M.7.2) that a treatment : background ratio of only 2:1 rather than one of 3:1 (SAUMWEBER et al. 1980, SAUMWEBER 1980) will be required when triplicates rather than duplicates are used to detect treatments differing significantly from background (assuming in this case that a probability of error of only 1% is acceptable). This example makes clear that the use of adequate replication coupled with statistical computation can be useful in extending the sensitivity of IRMA, but it should also be clear that these methods are no substitute for careful experimental technique. No amount of replication or statistical manipulation will ever allow a signal deriving from 10^7 ^{125}I atoms to be detected against a background of 1000 c.p.m. with an S.D. of 500 c.p.m.. However, if backgrounds can be reduced to about 100 c.p.m. with an S.D. of not more than 20 c.p.m., then replication can be used to push the limit of detection below 10^7 molecules of monoiodinated protein.

R.7.3.1.1.4 Limits of antigen detectability

The limits discussed until now are those imposed by the minimum amount of a **detector** of specific antigen-antibody complexes which can be "seen". In an indirect IRMA assay, one might theoretically expect to find several detector molecules attached to each antigen-bound antibody. Using polyclonal antibody, several antibody molecules might be attached to each antigen molecule. It is thus conceivable that the minimum number of antigen molecules which could be detected would be severalfold less than the minimum observable number of detector molecules. This remains a theoretical possibility. However, as I have shown in section R.7.2.1, unless both the first and second immune reactions use very high affinity antibodies, the proportion of antigen or immune complex which

binds antibody or detector may be only a small fraction of that present in each well. This question is addressed in sections R.7.3.4.1, R.7.3.4.2 and R.7.4.5, whereby it appears that many monoclonals may saturate binding to their antigens, but that this is not the case for polyclonal preparations, even when they are affinity purified. Empirically determined detector binding to antibody binding ratios under the assay conditions used up to now have always been less than unity (see R.7.4.5), i.e. on average **less** than one detector molecule is bound per antigen molecule. Thus, in quantitating antigen, the sensitivity limit imposed by detector "visibility" has still to be reached.

R.7.3.1.2 Range and limits of signal detectability in ELISA

R.7.3.1.2.1 Units of Measurement

The unit of measurement in an ELISA assay is a change in the difference between the optical density (O.D.) at two different wavelengths per unit time. One of the wavelengths is a reference wavelength at which the O.D. of the ELISA samples changes negligibly as the result of the enzyme catalysed reaction. This method of measurement allows the exclusion of errors arising from optical imperfections in the microtitre plates stemming, for example from small manufacturing defects or from small amounts of light scattering materials (e.g. dust) in the sample. The apparent "O.D." arising from such imperfections will be approximately equal at both wavelengths, if the wavelengths are not set too far apart, and their difference will then be zero, whereas the coloured product of the enzyme reaction absorbs differently at each wavelength and will be detected. Using empty "ELISA-quality" microtitre plates offered by several different manufacturers, the plate reader used in our laboratory gives a zero signal which fluctuates by at most 1 part in 1000, so that errors from this source are almost negligible.

Many enzymes can now be coupled to immunological reagents for ELISA assays: peroxidase, ß-galactosidase, glucose oxidase, alkaline phosphatase and a choice of substrates giving rise to coloured products for each are now commercially available. For the measurements reported here only alkaline phosphatase-coupled detection reagents and p-nitrophenyl phosphate as substrate was used. The arbitrary but practical units of measurement with which the assays were evaluated I will term "ELISA Units" or E.U. and were given by the equation:

$$\text{Assay Signal in E.U.} = \frac{O.D._{405} - O.D._{490}}{\text{duration of reaction in min.}} \times 1000$$

The use of the 405 nm and 490 nm wavelength pair for measuring p-nitrophenol is nearly ideal: the O.D. at 490nm is less than 0.1% that at 405nm.

R.7.3.1.2.2 Range of Measurement

The response of the measuring device is completely linear over the range 0 to 1.5 O.D. This range can be extended with very little error by measuring $O.D._{450}-O.D._{490}$ and converting it to its $O.D._{405}-O.D._{490}$ equivalent by simple calculation (see M.7.6). Thus measurement could be accurately made over the range 0.010 O.D. to approximately 8.0 O.D.. An O.D. of 0.010 set a lower limit, because even using digital signal averaging techniques, at very low signal values rounding and counting errors will start to become significant ($\pm$ 0.002 $O.D._{405}-O.D._{490}$ units). The time taken to reach these limits could also vary between approximately 15 minutes for the fastest reactions and approximately 24 hours (- 1500 minutes) for the slowest reactions, giving a potential range of measurement between $7\ 10^{-3}$ and $3\ 10^{2}$ E.U.. Measurement over periods much longer than 24 hours was precluded by the gradual inactivation of enzyme and non-enzymatic decomposition of the

substrate. However as the time course shown in fig. 30c illustrates, at low levels of enzymatic activity the rate of reaction remained nearly constant for at least 20 hours.

In some of the earlier experiments, the measuring device was incorrectly adjusted, with the result that the response of the machine became non-linear and unreliable at values exceeding 0.8 O.D.. This effect could also be compensated by measuring $O.D._{450}-O.D._{490}$, but the upper limit of measurement was then restricted to the equivalent of 5 $O.D._{405}-O.D._{490}$.

R.7.3.1.2.3 Non-specific binding background

The remarks in the section on IRMA backgrounds (R.7.3.1.1.2), apply to ELISA too. Since the concentration of detector used in ELISA is much higher than that used in IRMA, in general one might expect non-specific binding of detector to be a more serious problem than when using IRMA. However, using calf-thymus H1 as antigen and rabbit anti-H1 antibodies, it was possible to reduce background (antibody binding to BSA or detector binding to antigen) to the limits of detection (see R.7.3.4.3) by using 1.0 M NaCl and 0.1% Tween 80 in all the wash buffers subsequent to phosphatase binding. The choice of detergent was important, Triton X-100 at the same concentration resulted in substantial inactivation of the phosphatase enzyme (results not shown). My thanks to R. BABIEL for drawing the favourable properties of Tween to my attention.

However, even using Tween 80, non-specific binding of monoclonal antibodies to protein (e.g. BSA) posed a serious problem. High concentrations of some antibodies could cause the assay to become completely dominated by non-specific effects. This is illustrated in fig. 25 which shows the binding of a panel of mouse monoclonal antibodies to BSA under normal ELISA assay conditions. Note that, at concentrations greater than 1 µg/ml, all but one of the antibodies gives a

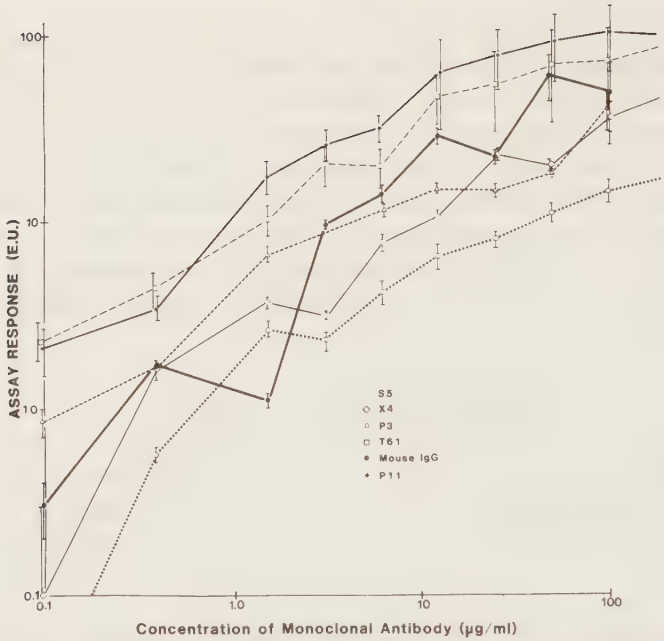

Fig. 25 **Non-specific binding of 6 monoclonal antibodies to BSA**
Double logarithmic plots of the assay response caused by non-specific binding of 6 monoclonal antibodies to microtitre plates coated with BSA, as a function of monoclonal assay concentration. Which curve corresponds to which antibody is indicated by the symbol key on the figure itself. Wells were coated with BSA by incubation with a 1 mg/ml solution thereof for 90 minutes. Incubation with purified monoclonal antibody diluted in PBS/0.1%BSA at the indicated concentrations was for 16 hours. The incubation with phosphatase coupled anti-mouse IgG was for 7.5 hours. All other details as described in M.2.5.2.3. The error bars indicate the 95% confidence limits of the regression (5 time points) from which the E.U. value was determined (cf. R.7.3.1.2.2). Individual time points are means of the values from quadruplicate wells and have been corrected for detector binding to the wells in the absence of monoclonal antibody, which was very small (<0.1 E.U.).

signal greater than 1 E.U.. Note also that there is as much as a factor 10 difference in the assay signal generated by each of the different antibodies: this may reflect differences in the efficiency with which the second antibody detects the monoclonal rather than in the propensity of the different monoclonals to bind to BSA (compare these results with

those in table 38 in R.8.3). To counteract interference from this source, it was necessary to work with low concentrations of monoclonal antibody (< 1 µg/ml, see R.7.4.4.1).

R.7.3.1.2.4 The effect of "blocking" with BSA

BSA was used in both IRMA and ELISA to block any binding of antibody or detector to protein binding sites on the microtitre plate well surface which, putatively, might remain free after antigen absorbtion. The aim was to prevent binding of either monoclonal antibody or detector directly to the well surface. However, if serum albumin (or other proteins) are included in the incubation buffers used in the assay, the "blocking" step, at least in ELISA, appears to be of minor importance, especially if protein antigen has already been bound to the well surface.

	Treatment Number			
	1	2	3	4
	No Antigen		Antigen	
	BSA Block	No Block	BSA Block	No Block
Detector Binding (E.U.)	0.20	1.42	20.1	20.6
(95% Confidence limits)	(0.04-0.37)	(0.70-2.15)	(17.1-23.2)	(18.4-22.8)

Table 25 **Direct Binding of Detector to polystyrene plates**
Microtitre plates were either left untreated or were coated with antigen (100 µl, 50 µg/ml mouse IgG, 30 min) and then either "blocked" with 300 µl BSA (0.5% w/v) or incubated in Borate-Saline (No Block). Thereafter the plates were washed and developed in the usual fashion (BSA containing buffers). Results are means of quadruplicate determinations.

The 7-fold higher value of treatment 1 than treatment 2 in Table 25 shows that BSA blocking does have an effect when all protein binding sites on the well surface are initially free, but even then only a small proportion of them (<2%) is

occupied by detector. Presumably protein in the washing and incubation buffers is sufficient to inhibit most detector binding. At signal levels whose attainment presumably requires that large areas of the well surface are covered by protein there is no statistically significant effect of blocking, as comparison of the results of treatments 3 and 4 in table 25 shows. Obviously "blocking" will be of importance when low amounts of antigen in very dilute solution are being assayed, but it is also apparent that aberrant ELISA responses with values > 5 E.U., such as those in the previous section, cannot be attributed to the trivial error of omitting the blocking step.

R.7.3.1.2.5 Non-uniform Assay response in microtitre plates: "edge effects"

The photograph of plate U in fig. 26 shows a rather extreme example of the "edge effect" phenomenon which bedevilled early attempts to develop a reliable ELISA assay. All the wells in this plate, with the exception of the leftmost column, have been identically treated. Furthermore, it is a blank plate, all wells have been blocked with BSA. It has not been exposed to antigen or monoclonal antibody, only to detector. No binding of detector should occur under these conditions. This expectation is fulfilled for the wells in the central portion of the plate, but there is a strong reaction in all the outermost wells and to a much lesser, but measurable, extent in some of the penultimate wells. The effect is caused by detector binding: blocking with BSA but omitting treatment with detector abolishes the effect, as the leftmost column on the plate illustrates (control experiments - not shown - in which this control row was placed in different positions on the plate, eliminated the possibility that this was a particular property of this position on the plate). Not only do the peripheral wells give high erroneous assay

responses (sometimes > 10 E.U), but the magnitude of the response varies in an unpredictable way from well to well.

It is clear that with variation of this magnitude in background values that it is going to be more or less impossible to measure specific antibody binding reliably. Plate S, shows how the edge effect is superimposed upon a specific antibody response (uppermost row of columns 2-10, compare with adjacent 3 wells in each column: the response in all four wells should be identical). The "edge effect" found in this experiment was particularly pronounced, and is shown for that reason. However, a particular difficulty encountered in trying to eliminate the effect was its lack of reproducibility. In some experiments the effect was only encountered on some plates and not on others. Using one batch of plates from a particular manufacturer which was used for many experiments it was possible to estimate that the effect occurred with a frequency of about one plate in every five, but the frequency clearly varied between plates from different manufacturers. Some of the curious properties associated with the effect are as follows:

1) Irreproducible

2) Variable magnitude both within (i.e between peripheral wells on the same plate) and between plates when it did occur.

3) Effect observed both in polystyrene and in PVC microtitre plates

4) Frequency of plates in which effect occurs varies with plates from different manufactures, and between batches from the same manufacturer (my thanks to the Firm of GREINER for providing several batches for test purposes).

5) Rotary movement or tilting movement of the plates during incubations did not reduce the effect.

6) Effect not observed, or very much less pronounced, in IRMA (at most a factor 2 difference between centrally and peripherally coated blank wells).

7) Detergent was present during the detector incubation

in IRMA but usually not the corresponding ELISA incubations. Control experiments (fig. 26 plate U) with tween 80 in the buffer used for incubation with detector did appear to reduce the magnitude of effect, but only by a factor of 2, still

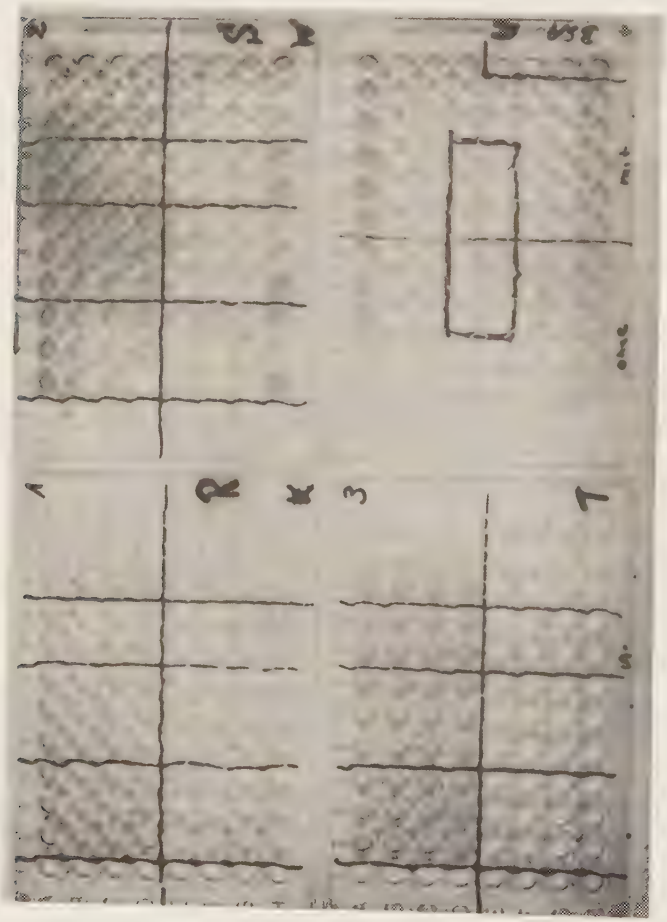

Fig. 26 "Edge effects": artefactual detector binding
(for legend see next page)

Fig. 26 **"Edge effects": artefactual detector binding**

Plate U: blank plate, all wells were blocked with BSA (0.1% w/v, 300 µl), washed and all except the wells in column (col.) 1 incubated overnight (16hr) at room temperature with detector (110 µl of a 1/500 dilution of phosphatase-coupled $(Fab')_2$ Rabbit anti-mouse antibody) in a covered but unsealed microtitre plate. Wells in columns 7-11 all rows and column 12 rows A-D contained 0.1% Tween 80 in the detector incubation buffer, all others did not. All wells were washed as usual (M.2.4.1.5) with tween containing solutions after the incubation with detector.

Plates R,S and T were treated identically, except where indicated. A culture supernatant containing 10% FCS and 40 µg/ml Q18/5/4 antibody was diluted with PBS by the factors 100, 300, 600, 900, 1200, 1600, 2000, 3000, 1200 or 1200. 4 100 µl samples of each dilution were used to coat rows A-D of columns 2-11 respectively. A series of dilutions of culture medium containing 10% FCS, but no antibody, made exactly as above was used to coat rows E-H of cols 2-11 respectively. All rows of column 12 were coated with 1/1200 diluted medium. Adsorption of antibody was for 23 hours at room temperature except for column 10 which was for only 3.5 hours. All wells including column 1 were blocked with 0.1% BSA for 1 hour (col. 10 overnight). All wells except col. 1 were incubated with phosphatase coupled detector as for 16 hours, as was plate U, except for col. 11 (4 hours). The incubation conditions for detector were as follows: after washing, plate S and T were incubated at room temperature, plate R on ice. Plates S and R were covered, but unsealed. The lid of plate T was lined with moist tissue paper and the plate and lid sealed with parafilm. All other conditions as detailed in M.2.5.1.4. Wells D2, E4, F4 and G4 on plate S were pipetted wrongly in setting up the experiment and should be disregarded. Other markings on the plates are irrelevant to the experiment described in the text.

leaving a manyfold difference between phosphatase binding to blanks in central and peripheral positions.

8) Effect was in general smaller when incubation with phosphatase-coupled detector was carried out on ice rather than at room temperature (compare plate R (ice) with plate S (room temp) in fig. 26. The specific binding of detector to antigen in plate R is less by a factor of approximately 2, but the reduction of the edge effect is proportionately greater (a factor 10 by quantitative measurement). The ratio between central and peripheral values is reduced to near IRMA levels. However the effect is still detectable by quantitative (ELISA reader) measurement.

9) Effect did not occur, or was negligibly small, when phosphatase coupled Avidin rather than phosphatase coupled antimouse IgG was used (cf. R.7.3.4.2).

10) Attempts to reduce temperature gradients over the plate during incubation (use of incubator, packing in ice) did not appreciably reduce the effect.

11) When substrate and phosphatase were premixed and distributed to all the wells of a plate the reaction occurred uniformly in all wells (data not shown). This observation would seem to exclude the possibility that different rates of convection driven mixing during the enzymatic reaction might be responsible for such effects.

The cause of the effect is, however, to a large extent trivial. Lining the plates with moist tissue paper and sealing the lids with parafilm eliminated the "edge effect", as plate T in fig. 26 shows (and as numerous subsequent experiments both by myself and M. FRASCH have confirmed).

I suggest that the explanation for the observed effects is then as follows. The plastic lids with which the plates are covered during incubations are somewhat loose-fitting. It would seem likely that the air above the peripheral wells might not have been saturated with water vapour and as a result evaporation might have taken place, leading to the deposition of phosphatase coupled antibody at the liquid air junction in some form which was particularly resistant to subsequent washing (I am indebted to M.FRASCH for this suggestion).

This hypothesis provides a plausible explanation of the variation between and within batches of plates from different manufacturers. All one has to suppose is that there are small variations in the dimensions of both lids (which were reused) and plates. If one further assumes that air movement under the lid is critically dependent on the gap between plate and lid, and possibly upon temperature, some of the observed effects seem slightly less mysterious. The very much smaller effect in IRMA may be explained as a combination of detergent and temperature effects (almost all IRMA assays were performed in the cold). The very much lower absolute concentration of ^{125}I

labelled detector compared to that of phosphatase labelled anti-antibody might also be unfavourable to its sticking.

Although this explanation of the edge effect is hypothetical, it has the virtue of being testable (measurement of plate dimensions, construction of deliberately loose-fitting lids etc.,). However, since it is now possible to eliminate the effect there seems little point in persuing its exact cause further.

Nonetheless, the hypothesis does leave one observation unexplained. Why is it that only phosphatase coupled antibody gives rise to this effect and not Avidin-phosphatase? Possibly the surface forces involved in forming an "unwashable" residue (?charge?) are different.

R.7.3.1.2.6 Empirical determination of signal detection limit

Because it was possible to sample the course of the enzymic reactions at different times, the following strategy was used to determine whether very low signals differed significantly from background. The reaction proceeded nearly linearly with respect to time. By sampling the difference between background and treatment signals at different times (sometimes 0 at early times) the best fit regression (=E.U.) of this difference on time and its S.D. could be calculated. It was then possible to test statistically (M.7.2) whether the regression differed significantly from zero (=background, by definition). In one experiment using H1-anti H1 (cf R.7.3.4.2), sampled at 7 time points, which gave rise to very low E.U. values, the empirically determined S.D. of a regression of 0.04 E.U. was 0.006 (or 15% of the best fit value). Using STUDENT'S t test it can be shown that a regression with these parameters differs significantly from zero at a level of P=0.01 (i.e. there is less than 1% probability on the basis of the measured scatter of the points that the observed deviation from zero could have occurred by

chance). Assuming that antibody binding producing still lower E.U. values would have a similar S.D. value, this would set a lower limit of 10^{-2} E.U. to the level at which a signal can be detected (with an error rate of 5%). This limit is only slightly more than that expected on the basis of the limits set by the reliability of the electronic signal detection and the maximum length of time for which the reaction may be followed (R.7.3.1.2.2), and indicates that no further improvement in signal to noise ratio could be obtained in this particular assay.

R.7.3.1.2.7 Comparison of ELISA and IRMA sensitivity

It is not possible to equate an E.U. simply with a certain number of detector molecules as it is the response in the IRMA technique. There are too many unknowns which determine the the relationship between E.U. and antibody. It is not a practicable proposition to produce enzyme-labelled anti-antibody in small amounts. Production of highly active enzyme coupled anti-antibody in reasonable yield is only feasible (FLEHMIG pers. comm.) if large amounts of anti-antibody are available (100 mg+). Since such large amounts of material were not available during the period in which I was experimenting with ELISA, it was necessary to rely upon preparations from commercial manufacturers. The activity of coupled antibody supplied for the same price varied wildly from manufacturer to manufacturer (< factor 100), setting limits on the dilutions which it was economic to employ. The specificity and degree of substitution of detector with enzyme was unknown. It is usually not possible to determine the amount of specific antibody in such preparations, because they usually contain non-specific carrier antibody to stabilise the preparation, and in any case, the spectral properties of the conjugated IgG differ from that of pure IgG. There certainly appeared to be some differences in specificity between the

most commonly employed IRMA antibody and that used in ELISA (cf. R.8.3). The temperature at which the enzyme reaction takes place can be varied and phosphatase might be bound to antigen either directly via anti-antibody as in the IgG assays or indirectly via two intermediates in the phosphatase-avidin/ biotinyl anti-antibody combination as in the anti-H1 assays.

However, using the same antigen, it is possible to compare a particular ELISA technique, using a particular detector, with IRMA. For instance, it is possible to calculate from the results shown in section R.7.3.5 that in the H1 assay system under identical conditions (i.e. P.V.C. plates, identical concentrations of antigen and antibody), that 2 E.U. would be equivalent to the binding of approximately 10^5 c.p.m. (10^{10} molecules) of a monoiodinated detector protein. This means that the lower limit of signal detection of ELISA (10^{-2} E.U.) is approximately 5 fold higher than that of IRMA (5 10^7 compared to 10^7 detector molecule equivalents). For most practical purposes, this is no hinderance, since the use of higher concentrations of antibody and detector molecules will more than compensate for this small intrinsic difference in sensitivity (R.7.3.4.2).

R.7.3.2 Specificity, Activity and Specific Activity of Iodinated Detector Molecules

Obviously the specific activity of labelled protein is going to be one of the factors which determines the sensitivity of IRMA.

The rabbit antibodies used to detect H1 bound to solid phase were visualised by I^{125} labelled protein A (WELSH et al., 1975, BIBERFELD et al., 1975). With rabbit IgG (but not with mouse IgG) protein A has a uniform reaction and a high affinity constant (K_D 10^{-8}, cf. table 19 p.203). There has been some discussion in the literature about the effectiveness of various methods of iodinating protein A and of possible inactivation as the result of derivatisation (LANGONE, 1982a,

MAYERS and KLOSTERGAARD 1983). For this reason I describe the properties of the iodinated protein in some detail.

Rabbit anti-mouse Fab was chosen as a reagent for the detection of mouse antibody because it should (and has), through its reaction with light chain determinants, allow(ed) recognition not only of IgG but also of all other Ig classes (e.g. IgM). Since the various subclasses of IgG are very unequally represented in mouse sera and since anti-antisera are often thought to be directed against subclass specific epitopes in the Fc region, it was hoped that the use of an anti-Fab antibody might give a more uniform quantitative response than anti-whole molecule antibody which might be biased toward recognising one particular subclass.

R.7.3.2.1 Iodination and IgG binding ability of Protein A

The various iodinated protein A preparations used in this work were labelled at a molar ratio of about 1.0 (mole I/mole protein, range 0.7 - 1.55 , 7 preparations), corresponding to a specific activity of about 50 µCi/µg or 2000 Ci/mmole using the chloramine T method (see M.5.1). However, even when the solid phase was saturated with immune complexes in amounts greatly exceeding the amount of labelled protein A in the system, at most 60% of the labelled material was able to bind to the IgG, even after 24 hours incubation ($0^{o}C$) .

The reason why 40% of the molecules in the protein A population are unable to bind IgG is not known. The material obtained from the manufacturer (Pharmacia) was not homogeneous. Although a major band was present in SDS-PAGE only 50% of the ^{125}I incorporated was found there. The remaining material was smeared in the region both above and below the major band and in addition there were some discrete bands in the high M.W. region of the gel (not shown). A part of the extraneous material could be removed by size chromatography on Biogel, however this did not increase the maximum proportion

of label which could be bound to the solid phase, suggesting that the extraneous material may still represent biologically active protein A derivatives (e.g. active degradation products in the low molecular weight region and oligomers with MWs greater than the main band in the higher M.W. region).

MAYERS and KLOSTERGAARD (1983) have iodinated protein A (obtained directly from Pharmacia and used without any further purification) using a variety of methods. They also found that at most 60-70% of their iodinated material was capable of binding to rabbit IgG, regardless of the method of iodination. When chloramine T method was used the proportion of protein A which was active was relatively **insensitive** to the mole I/mole protein ratio in the range 1 to 9. The conditions of exposure to chloramine T also do not appear to be critical: I was able to obtain 80% incorporation of label into protein after 20s on ice, whereas MAYERS and KLOSTERGAARD used 5 min. at room temperature at a similar concentration of chloramine T. These results would appear to exclude the possibility that the 40% of labelled protein which is unable to bind IgG arises as a result of chemical inactivation during iodination under these conditions. This does not necessarily mean that protein A is insensitive to chloramine T: the concentration of chloramine T used by MAYERS and KLOSTERGAARD and myself is approximately 100x lower than the concentration usually used for iodinating proteins (HUNTER and GREENWOOD 1962) and it has been reported that use of the latter conditions does inactivate the molecule (DORVAL et al., 1975).

Chloramine T causes the iodination of both tyrosine and histidine. Protein A has 4 equivalent binding sites for IgG, only two of which are operative at any one time, and both histidine and tyrosine are thought to be essential for binding activity (SJÖHOLM et al., 1973; DEISENHOFER 1981). Since there are only 8 such residues per molecule (SJÖHOLM and SJÖDIN 1974), the finding that as many as 9 molecules of iodine can be incorporated per molecule without drastically affecting function is a little suprising, especially since two other

oxidative iodination methods, using peroxidase or periodate, do appear to result in inactivation when protein A is substituted at molar ratios greater than unity. MAYERS and KLOSTERGAARD have presented evidence which suggests that this difference may be explained by the ability of chloramine T to preferentially cause the diiodination of tyrosine and possibly also of histidine residues, whereas the other techniques do not.

There have been a number of reports (LANGONE 1982a, WILDER et al., 1979), which describe labelling of protein A at residues other than tyrosine. In a study using protein A modified at lysine residues by ^{125}I Iodotyrosine using Bolton-Hunter reagent, a specific activity of the protein only about 10% of that which I usually obtained (LANGONE 1978) was achieved. Even at this low level of modification to residues which are not thought to be involved in IgG binding the maximum proportion of molecules which could be bound to solid-phase bound antibody was 50%. MAYERS and KLOSTERGAARD (1983) also report disappointing results with this reagent. However ^{125}I protein A labelled using Bolton-Hunter reagent and with a specific activity of approx. 1300 Ci/mmole is now commercially available (Amersham). The most promising reagent examined by MAYERS and KLOSTERGAARD (1983) was WOODS reagent, use of which makes it possible to label protein A at many lysine residues with diiodobenzamidine. Even higher specific activities (>500 μCi/μg) than those obtainable using chloramine T were obtained and a higher proportion (90%) of the labelled material was able to bind IgG.

To conclude, although it clearly is possible to inactivate protein A using conventional iodination techniques, it is unlikely that this was the cause of the inactive fraction which I observed. The procedure which I used was exceptionally mild compared to that used by other workers, and yet their preparations contained similarly large inactive fractions. There are at least two alternative explanations. Firstly, the material supplied by Pharmacia might contain

material which is inactive. Secondly the factors discussed in section R.7.2 could play a role. Because of its shape (LANGONE 1982a), Protein A has the same diffusion coefficient as IgG. This means that when non-saturating amounts of this protein are used, even after 24 hours one would not expect more than 90% of the material in the well to have been transported to the vessel walls (cf. fig. 23, p.234). In addition, if the equilibrium binding position for protein A in homogeneous phase can be extrapolated to solid phase, then not more than about 80% of the active I^{125} protein A in the system would be expected to bind anyway (cf. fig. 20 p.208). If these two effects (transport and binding equilibrium) are additive, then the maximum amount of protein A which one would expect to bind would be about 70%, close to that found experimentally.

The two suggested explanations (inactive starting material and equilibrium considerations) are of course not mutually exclusive. If either (or both) of them is (are) correct, the problem now arises of how the 90% binding of the protein A modified by WOODS reagent (MAYERS and KLOSTERGAARD 1983) can be explained. If the observation of these authors can be confirmed, it opens up the intriguing possibility that this modification might increase the affinity of the molecule. In any case, even the very high specific activity claimed to be possible with WOODS reagent, would make it interesting to investigate the properties of these derivatives and suggests that it might be possible to increase the sensitivity of assays using radiolabelled protein A by as much as factor 10.

R.7.3.2.2 Iodination and Binding Specificity of RAM-Fab

The production and iodination of the RAM-Fab used in IRMA has been described elsewhere (RISAU et al., 1981). As was the case for iodinated protein A, the mean level of iodination was 1 mole I^{125}/mole protein.

The maximum amount of binding of this material to non-

limiting amounts of commercially obtained "mouse IgG" was only about 15 - 25% of the counts used in the assay. Similar efficiencies were also observed against some purified monoclonal antibodies (see R.8.3). Although a proportion of the RAM-Fab might have been inactivated either by iodination itself or by the relatively harsh treatment which was used in eluting it from the affinity column which was used for its purification, it seems improbable that 90% of the material was inactive. An observation which supports this contention is that when the Tübingen RAM-Fab was directly compared with a commercially available iodinated RAM-Fab (AMERSHAM) in this same assay it performed at least as well, if not better. However, the manufacturer claims that his material is 60%-70% active in binding to IgG attached to Sepharose beads.

There are two possible explanations which might explain this discrepancy. Firstly, it is possible that both the assay used by the commercial manufacturer and the affinity procedure used in Tübingen allows the detection (isolation) of antibodies with low binding constants ($< 10^6 \ M^{-1}$) and that these make up a substantial proportion of the RAM-Fab population. These antibodies must be able to bind in the columns used for their affinity purification, but the effective concentrations of IgG and anti-IgG in such columns are probably very high compared to those used in solid phase assay. Alternatively, if the "mouse IgG" against which the RAM-Fab was usually tested only had limited heterogeneity, as IEF analyses indicated (some manufacturers may be selling myeloma- rather than serum-derived IgG), only a small proportion of the RAM-Fab population (raised against authentic serum IgG) might bind to that particular set of antigenic determinants (cf. R.7.2.5).

Binding efficiencies of this order (20%) have been noted by a number of other authors who work with iodinated anti-mouse IgG antibodies, most explanations that have been offered assume that iodination damage is responsible for these low efficiencies, but no convincing data has been produced to support this contention (see e.g. PARHAM et al., 1982).

R.7.3.3 The time course of binding to solid phase: correlation with diffusion kinetics

Fig. 27 shows the time course of binding of a limiting amount of histone H1 (50ng) to the surface of a polystyrene microtitre plate well. An H1 solution was placed in the wells and, after the indicated periods of time, the wells were aspirated and the H1 solution replaced by a solution of BSA. The wells which were exposed to H1 for the shortest period of time were also exposed to BSA for the longest period of time, hence a formally possible explanation for the low level of binding after short periods of incubation with H1 might be that it was the result of the long period of exposure to BSA (e.g. competing away previously bound H1 molecules), rather than the exposure time to H1 per se. However, this possibility can be eliminated, because the same result was obtained when the incubation with the H1 solution was started at different times such that the length of incubation with BSA was kept constant (not shown). A corollary of the latter result is that it demonstrates that the signal generated by antigen adsorbed to the walls does not change with time. This implies that the antigen which can be detected immunologically is essentially irreversibly bound (cf. also fig. 23).

The heavily drawn curve through the experimental points in fig. 27 is a theoretical one for the diffusion of molecules to the walls of a completely absorbing sphere of equivalent volume to a microtitre well, for which the exponent term in the computational algorithm (M.7.5) has been corrected by a factor 0.7 to simulate the condition that only 70% of the boundary area of the liquid in a well makes an interface with an adsorbing solid phase. The two lightly drawn curves show the expected rate of diffusion out of a sphere for which no such correction has been made (upper line) and for a cylinder of equivalent radius and with perfectly non-adsorbing ends (lower line).

The experimental data fits the heavily drawn theoretical curve very well.

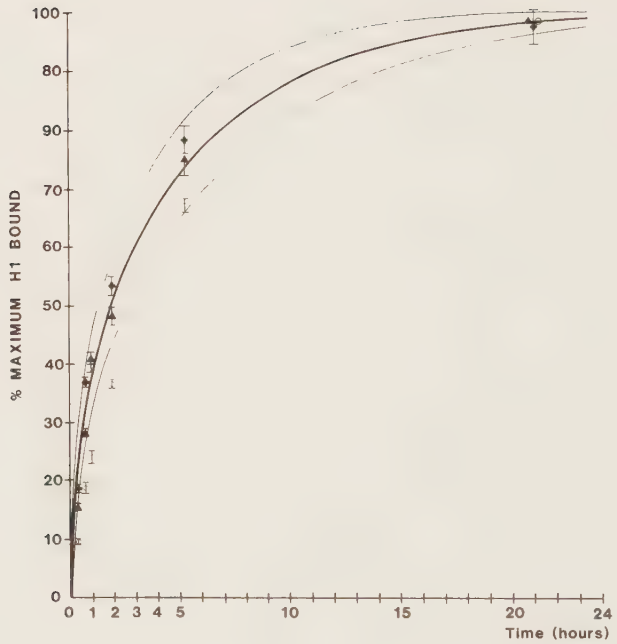

Fig 27 **Time course of H1 binding to polystyrene microtitre wells.** A solution containing 50 ng H1 was incubated in the wells of a polystyrene microtitre plate for the lengths of time indicated on the abscissa. The wells were then aspirated and the H1 solution replaced by a solution containing 0.1% BSA until the longest H1 incubation had been completed. Immune complexes were formed on the bound H1 by incubating the wells with either of 3 different anti-H1 antibody preparations (R.4.1), 147 (+), 402 (▲) and 405/6 (○), each at a concentration of 10 µg/ml, for 19 hours. Immune complexes were visualised using biotinyl goat anti-rabbit antibody (1/200 dilution, 6hr) and avidin-phosphatase (1/200 dilution, 2hr) as detector. All incubations were carried out at room temperature. Other details as outlined in section M.2.5.2.1. The amount of H1 bound has been estimated by assuming that the assay signal, in E.U., is directly proportional to the amount of adsorbed H1 and normalised by assuming a value of 95% for the 21 hr time point. The extent of substrate hydrolysis by phosphatase in duplicate wells was measured at between 5 and 7 time points and, after correction for non-specific binding (subtraction of values from replicate wells to which only BSA

(continued on bottom of next page)

I will argue in section R.7.3.5 that the signal which is generated by adsorbed antigen is directly proportional to the proportion that has been physically transferred to the vessel wall, although the constant of proportionality may differ between different plate types or adsorbtion conditions. This contention does not contradict the interpretation of the time course given here, provided that the immunologically detectable fraction of the initially bound material remains constant. The possible exceptional case which I discuss below illustrates the problems which may occur if this condition does not hold.

On PVC plates, using IgG as antigen, serial transfer experiments confirmed the correctness of this assumption (R.7.3.5.1). The kinetics of binding by IgG to PVC plates, both in the warm and in the cold, measured using IRMA, conformed to theoretical expectations within the limits of experimental error (R.7.3.5.1 and results not shown).

There thus appears to be fundamental agreement on the time course of antigen binding using both different antigens and detection methods.

I have a single set of data (not shown) which does not agree with the diffusion hypothesis. In this experiment a limiting amount of H1 was bound to PVC plates at room temperature and a maximal assay response was observed after only 6 hr. It is difficult to explain this result. Possibly

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(Legend to fig. 27 continued from previous page)
had been adsorbed but which had otherwise been treated identically), E.U. values were calculated as described in M.7.6. Theoretical curves were calculated as described in section M.7.5. The thick line shows the theoretical curve for diffusion to the wall of a sphere with 70% of its surface absorbing, the thin line above it diffusion to the walls of a sphere with 100% of its surface adsorbing and the lower thin line that to the walls of a cylinder. A value of $5.5\ 10^{-7}$ $cm^2\ s^{-1}$ has been assumed for the diffusion coefficient of H1. Vertical bars show the 95% confidence limits. For strict comparison 2 other sets of experimental points, normalised with respect to the 21 hour values of thin line curves, should be shown. This comparison has been omitted, because the position of the experimental points thus estimated are only negligibly shifted with respect to those shown.

the hydrodynamic system in the well is sufficiently metastable that convective transport does sometimes occur. This is a most unattractive possibility, since it suggests that such "aberrant" results might recur with a frequency which might be unpredictable unless large scale statistics were kept. An alternative and almost equally unattractive hypothesis is that the immunologically detectable fraction is a variable fraction of the physically transferred molecules under this particular set of conditions. Unattractive though this idea may be, it is plausible. Table 26 (R.7.3.5) shows that the maximum signal generated by 50 ng of H1 on binding to PVC at room temperature is only 70% of the maximum signal obtained when this amount of H1 is bound to PVC in the cold under the same conditions. Reference to fig. 27 shows that 70% is precisely the proportion of H1 that one would expect to be transferred to the vessel walls after 6 hr. Thus if the proportion of H1 which remained immunologically detectable after having initially bound there were to fall from nearly 100% at short times of H1 exposure to only 70% after 21 hr exposure, the unfortunate situation might occur in which the increase in signal which would be expected from increasing amounts of transferred H1 would be almost exactly offset by a process (?denaturation of determinants?) rendering it immunologically undetectable.

In summary, most of the experimental observations I have made are consistent with the idea that, in vessels of micro-titre plate well dimensions, under the experimental conditions used in our laboratrories, transport from bulk solution to the walls of the vessel is an exclusively diffusive process. I feel that I have presented sufficient evidence to back this claim. However, at a formal level the explanations which I have presented to explain inconsistent data require further measurement, which I was unfortunately not able to perform.

R.7.3.4 Effect of Antigen and Antibody concentration on assay response

Section R.7.3.4 shows how the signal generated in either IRMA or ELISA varies as a function of antibody and antigen concentration using defined amounts of purified antigen and antibody. The results are used to derive estimates of the maximum sensitivity of each kind of assay, and in the case of ELISA to illustrate the range of measurement that is possible.

The IRMA titrations which provided the data used in this section were not performed with this end in mind. A consequence of this is that the data upon which the IRMA sensitivity estimate must be made has to be extrapolated. What assumptions are justified in making the extrapolation are argued in detail. Detailed argument is neccessary because the resultant estimate is highly dependent upon the efficiency assumed for either antibody or detector. The ELISA data is more complete and covers a wider range (100x larger) of concentrations than the IRMA data, but is not entirely satisfactory. Close scrutiny of the ELISA data and comparison of the ELISA and IRMA data suggests that the binding of the anti-H1 antibody in both kinds of assay, but not of IRMA detector, is very inefficient. This inefficiency occurs even though the antibody has been purified by affinity chromatography. Possible reasons for this inefficiency are discussed.

R.7.3.4.1 In IRMA - H1 as antigen, protein A as detector

The detection limits of this IRMA system for antigen have not been directly determined, because the assay system was used to detect and quantify antibody rather than antigen (see R.2, R.4). The results shown in fig. 28 indicate that it was suited to this purpose: the assay response to limiting amounts of antibody is linear and unaffected by the antigen concentration used to coat the plates over 5 orders of magnitude.

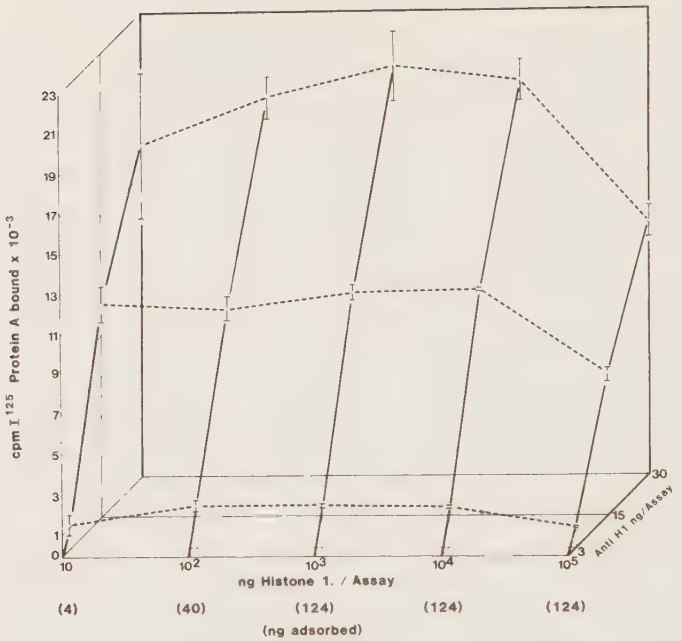

Fig. 28 **Response surface of I^{125} protein A binding in IRMA assay of H1 titrated with respect to amount of antigen and antibody.**

Conditions of assay were as described in section M.2.5.1.2. Antigen was adsorbed to PVC in 100 µl volume for 90 min. on ice. The anti-H1 antibody preparation used for immune complex formation was 405/6 (cf. R.4.1). 33.5 fmole of I^{125} protein A (sp. activity 1.73µCi/pmole, = 93,000 cpm) was used to detect immune complexes. Each vertical plane in the figure has been drawn to the same scale. The error bars indicate the range of duplicate measurements. The upper scale on the abscissa ("ng H1/assay") indicates the actual amount of H1 placed in each well. The lower scale ("ng adsorbed") shows the maximum amount of H1 which could diffuse to the well walls during the incubation period used.

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The largest amount of antibody or antigen which could be determined would be limited by the amount of detector used in the assay (see introduction to section R.7). The lowest amount can be determined by some plausible extrapolation of the data

in the figure. At the lowest two concentrations of antigen used, the amount of H1 was less than the total binding capacity of the well surface (see R.7.3.5), so the amount of antigen bound to the plate was certainly different at these concentrations. This allows some conclusions to be drawn about the characteristics of the assay and about the minimum level of antigen which can be detected in the assay.

The wells were incubated with the H1 solutions for 90 min in the cold: at most 40% of limiting amounts of H1 in the wells would be able to diffuse to the walls in this period of time, implying that, at the lowest concentation of H1 used, at most 4 ng H1 (200 fmole) was actually bound to the walls of the wells (this would give a concentration of $2.0\ 10^{-9}$M if it were free in solution in the well). The highest amount of anti-H1 used (30 ng/well =130 fmole at a concentration of $1.3\ 10^{-9}$ M) was less than the amount of adsorbed H1 on a molar basis, so that H1 is not expected to be limiting. ELISA results (see next section) indicate that only a small fraction of the total antibody is likely to be bound. However, because the assay response is independent of antigen concentration, this implies that the fractional part of the 405/6 antibody preparation that is binding must be sufficiently affine to be completely bound at the lowest concentration of antigen. It is not possible to estimate this fraction from the available data, but it is possible to set some limits to it.

At the one unlikely extreme, if all the anti-H1 were able to bind, then the 5.4 fmoles of detector bound at the highest anti-H1 concentration would correspond to the equilibrium position expected for protein A binding in homogeneous phase conditions (binding efficiency approx. 5%, calculated by treating the adsorbed H1 as if it were free in solution). If the binding effiency of protein A is higher than in solution (which seems likely, data not shown), then, at the other logical extreme the bound I^{125} protein A might binding all the anti-H1 present in the well. This places a **lower limit** of 4% on the proportion of high affinity anti-H1 in the 405/406

antibody preparation. Thus, at one extreme it is possible that 96% of the 405/406 preparation might be incapable of forming detectable complexes with H1 at the concentration used for IRMA. At higher antibody concentrations ($7\ 10^{-9}$ M, 70x that used in the IRMA experiment shown in fig. 28), the amount of ^{125}I Protein A bound is clearly dependent on the amount of adsorbed H1 in the assay (in contrast to the independence in this IRMA assay), suggesting that it is low affinity of much of 405/406 antibody which prevents it from being seen in IRMA when it is used at low concentrations.

Binding of only 4% of the 405/406 antibody preparation is a limit value, the actual amount is likely to have been somewhat higher and to have been offset by a less than quantitative I^{125} protein A binding. Whatever the precise stoichiometries, to be able to bind completely at the lowest antigen concentration, the fraction of the anti-H1 that did bind must have had an affinity (avidity) on the order of $10^{10}\ M^{-1}$ (cf. fig. 20).

It must by now be clear that both the anti-H1 and detector concentrations used to obtain the results shown in fig. 28 were suboptimal, perhaps by as much as a factor of 10. Nonetheless, because of the fraction of highly affine antibody in the preparation it was possible to observe a signal of $1.8\ 10^{4}$ cpm from only 4 ng of antigen at an antibody concentrations that arguably might have only given only 4% of the potentially available immune complexes. Bivalent antibody binding is unlikely to account for a substantial proportion of the observed signal in view of the constant signal observed at very different antigen densities (see R.7.2). A signal of 500 cpm would still be clearly observable. Even assuming that only as much as 10% of the 405/406 antibody preparation were able to bind it would thus be reasonable to expect, using very much higher antibody concentrations, (approx. 100x greater) that a detectable signal could be obtained from about 1/400 ($500/2\ 10^{4}$ x 10, minimum detectable signal/observed bound x suboptimality correction) the lowest amount of antigen shown

in fig. 28 i.e. about 10 pg, 0.5 fmoles or 3.0 10^8 molecules of H1. This is somewhat better than the theoretical predictions (R.7.2.1.3) mainly because the binding properties of protein A appear to be better than expected. Increasing the concentration of detector used in this particular assay, providing the problems of non-specific background, waste and safety could be overcome, would be expected to raise assay sensitivity, but not necessarily proportionately. What increases in signal strength higher detector concentrations could achieve practically would have to be empirically determined.

To reach the sensitivity limit, using a quantity of solution containing only the limit amount of antigen mixed with other proteins, the antigen would have to be allowed to adsorb to the wall for many hours. Furthermore the proportion of antigen, relative to other proteins in the mixture, would have to be sufficiently high (on the order of 0.01% or 1 part in 10^4 under assay conditions using 10^5 cpm protein A as detector) to ensure that the solid phase did not become saturated by the other proteins before all the antigen was able to bind.

R.7.3.4.1.1 Effect of salt on assay response to pure H1 in solid phase assay

When sonicated nuclei are used as a source of H1 as antigen, salt increases the assay signal (R.7.4.2). The results presented here, using pure H1 as antigen, suggest that the cause of the increase is not directly attributable either to an increased capacity of the well surface for H1 or to an increase in the affinity of anti-H1 for its antigen.

When a titration such as that shown in fig. 28 was performed with buffers at all steps (including the antigen adsorption step) adjusted to either 0.7 M or 2.0 M NaCl, the assay response was in general decreased. When 10 ng H1 was used to coat the wells the decrease in assay response was by a

factor 2-3 (fig. 29a). When a potentially saturating amount of H1 was used (1000 ng) the decrease is assay response was less, only 20% (fig. 29b).

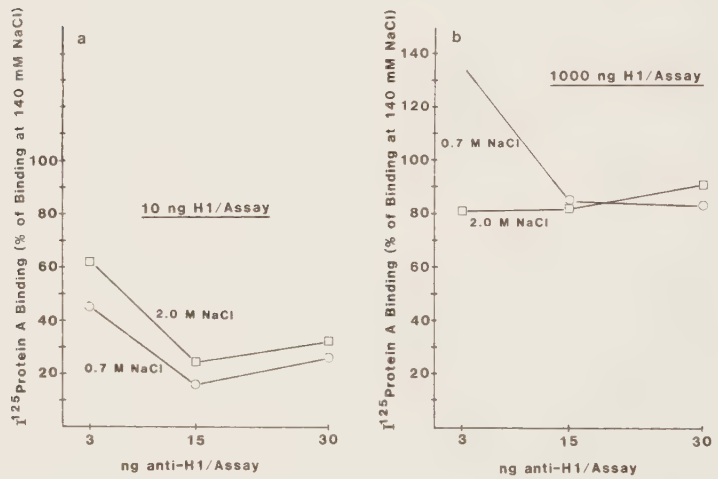

Fig. 29 **Effect of salt on IRMA assay response to pure H1**
IRMA titration performed in parallel with that whose results are shown in fig. 28, except that the indicated concentrations of NaCl were used at all stages of the assay. Results are shown as % of the assay response obtained using H1 in PBS (except for washes following the antibody-antigen reaction). Panel a), 10 ng H1 used to coat each well. Panel b), 1000 ng used to coat each well. Means of duplicate values are shown. Other conditions see legend to fig. 28.

There are at least 3 possible mechanisms and combinations thereof which may have contributed to this decreased response.

i) An altered diffusion rate to the plate surface (unlikely, might arise if H1 were to undergo massive conformational change)

ii) Altered retention of initially bound H1 (cf. R.7.3.5.2)

iii) Alteration of the K_{eq} of the H1-anti-H1 reaction.

Under the conditions of titration these 3 effects would are confounded, so it is difficult to decide to what extent each might contribute. Mechanism (ii) would be expected to be independent of antigen concentration and is therefore only likely to have contributed as much as the reduction at saturating H1 concentrations.

The exact mechanism is not important. What is important about these observations is that they exclude the possibility that salt increases the capacity of plate wells for binding H1 per se. An increased affinity of antibody for H1, at least for the 405/406 antibody is also excluded. These results suggest that the explanation for the effects described in section R.7.4.2 must be sought elsewhere than in the basic solid-phase-antigen-antibody interactions.

R.7.3.4.2 Effect of antigen and antibody concentration on assay response. In ELISA - H1 as antigen: biotinyl anti-rabbit IgG and Avidin-phosphatase as detector

Fig. 30 shows the response surface of an anti-H1 preparation (147) binding to H1 in ELISA. Apart from showing that this multistep assay procedure is capable of giving reasonable results, it also shows the ability of ELISA to allow measurement over a wide range not only of antigen amounts but also, more importantly, of antibody concentrations. The dashed line labelled "IRMA-meas" to the right of fig. 30 shows the highest concentration of antibody used in the IRMA titration shown in fig. 28. The line labelled "IRMA-sat" in fig. 30 shows at what concentration of antibody the IRMA assay would have become saturated because of limiting amounts of detector. Unfortunately, the amount of one of the detector components (biotinylated anti-rabbit antibody) was not saturating at high antigen and antibody concentrations. Controls showed that, using the highest concentration of H1 and anti-H1, increasing the concentration of this detector component by a factor 4 resulted in a increase in the assay

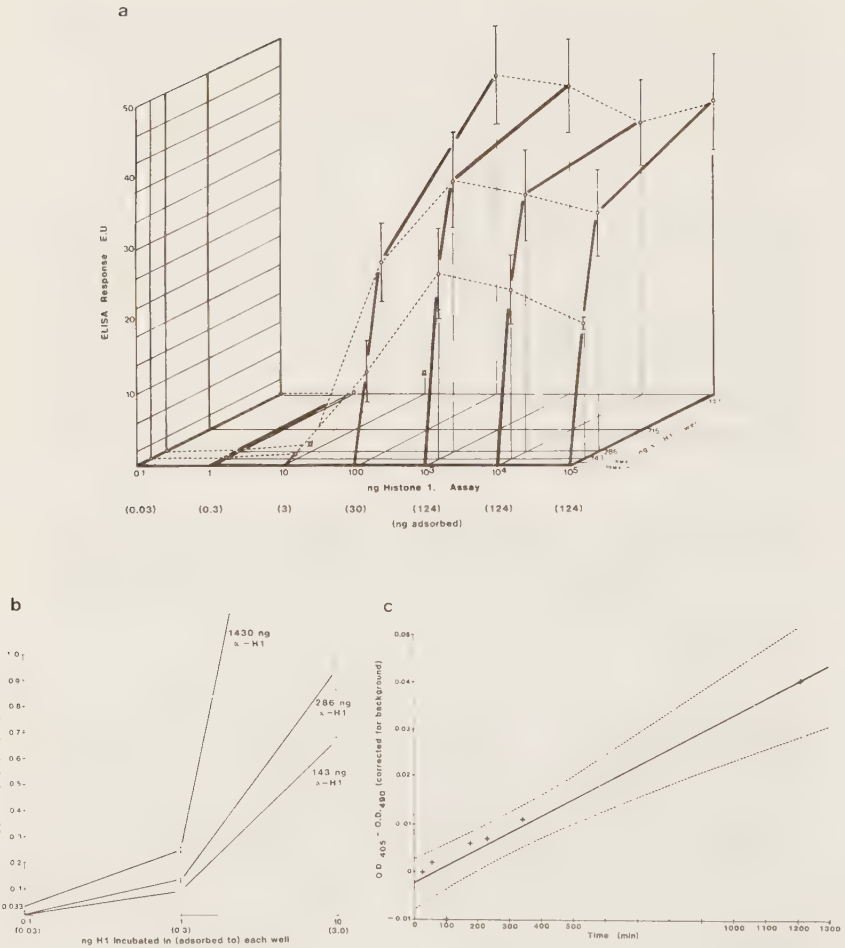

Fig. 30 **Response surface of ELISA assay titrated with respect to the amount of antigen H1 used to coat the plates and to the amount anti-H1 used to detect bound antigen.**

The assay was carried out in polystyrene plates. The amounts of H1 indicated by the "ng histone 1/assay" scale on the abscissa were placed in the plate wells for 30 minutes at room temperature, during which time the maximum amount likely to have absorbed is indicated by the "ng adsorbed" scale. The assay was performed as

(continued at bottom of next page)

response by at most a factor 1.7. Avidin concentrations on the other hand were saturating. Thus, the results shown in fig. 30 at the highest H1 and anti-H1 concentrations (right hand side of the rearmost plane) are somewhat misleading, since they would have been higher if adequate amounts of detector had been used. Inadequate detector concentration may also be the explanation for the apparent saturation of the assay response in wells incubated with 100 ng H1 and high concentrations of anti-H1. This result is almost certainly erroneous. 50 ng H1 given sufficient time to adsorb completely was shown in a number of subsequent experiments to be unable to saturate the assay response, but since the H1 for the results shown in fig. 30 was only incubated in the wells for 30 min at room temperature at most 30% (i.e. 30 ng, insufficient to saturate the assay) of the the material in the well could have been transferred to the walls by diffusion (cf. R.7.3.3).

The confidence limits of the higher E.U. values shown in fig. 30 are large. This is attributable, at least in part, to the sampling of only a few time points with an accuracy of only $\pm$ 1 minute with respect to time. Obviously this sort of accuracy can lead to a large error in the determination of reaction rates at early times (< 5 min) and if only 4 time points are sampled to obtain an estimate of the enzyme reaction rate, the early time value may contribute over-

- -

(Legend to fig. 30 continued from previous page)

described in M.2.5.2.1, using the 147 (M.4.1) anti-H1 preparation. Panel a shows the response surface: the dotted lines running in the same direction as the abscissa describe portions of the response surface which are concave (i.e. would be hidden from view if the response surface were opaque) the dashed lines which are continuous with them show the contour of the convex part of the surface. Panel b shows the lefthand portion of panel a in detail. Panel c shows the raw data from which the E.U. assay value for the least amount of detectable antigen was obtained (0.1 ng H1, 1430 ng anti-H1/well). The values in panel c have been corrected for non-specific binding of the the anti-H1 preparation and of detector. Data points in panel c are means of duplicate treatments. The error bars in panels a and b show the 95% confidence limits for the E.U. values (cf. M.7.6) determined from between 3 and 7 time points.

proportionately to the error estimate. Sampling more time points more accurately (with respect to time) should remove this source of error.

The ELISA titration of antigen, in contrast to the one using IRMA, shows that, when sufficient antibody and detector is used, 3 ng adsorbed H1 (150 fmole, 10 ng H1 incubated in a well for 30 min) is insufficient to saturate the assay response. The ELISA titration with respect to anti-H1 suggests, similarly to the IRMA results, that, at all concentrations of antigen and antibody at which detector is not limiting, the assay response is approximately linearly dependent on antibody concentration, although the slope of the line describing this dependency falls off markedly at low amounts of antigen. The ELISA signal obtained when 143 ng anti-H1 (at a concentration of 10^{-8} M) is incubated with at most 3 ng adsorbed H1 is thus approximately 10 fold smaller than the signal generated by 1430 ng anti-H1 (at 10^{-7} M). This indicates that at most 10% of the antigenic determinants on the adsorbed H1 can have bound antibody at the lower antibody concentration. 143 ng of anti-H1 is a 10 fold molar excess with respect to absorbed H1. Thus if the anti-H1 is able to bind H1 at only a single antigenic determinant, as the immunoprecipitation (R.5) and Drosophila H1 crossreaction (R.11.1) results might suggest, this implies a binding efficiency of the 147 anti-H1 preparation of at most 10% (with respect to antigen), even though the antibody is in molar excess. Only 1%-2% of the total antibody present in the well is able to bind. Under homogeneous phase conditions this would suggest a K_{eq} for the 147 anti-H1 preparation of $<10^{7}$ M^{-1} (cf. fig. 20). If the 1%-2% bound antibody were to represent a limiting amount of a high affinity subpopulation in the preparation, then the affinity of the bulk of the antibody in the preparation might be still lower. The assumption of a single antigenic determinant is probably an oversimplification. If more determinants were reactive, the efficiency of antibody binding would be less and a molar excess of antibody with

respect to the total number of antigen molecules need not represent a molar excess with respect to particular determinants. On the other hand, the estimate of 3 ng immunologically detectable H1 adsorbed to polystyrene is probably also an overestimate by a factor of about 2 - 3 (cf. R.7.3.5) and this would at least partially offset any increase in antibody binding efficiency which might result from multiple determinants.

The antibody preparation used for the IRMA titration, 405/6, gives an absolute assay response on PVC plates which is similar to that of the 147 preparation when high concentrations of H1 and antibody are used, (cf. table 26 in section R.7.3.5.2) but at low concentrations of antigen (<10 ng on PVC plates) the ELISA signal using the identical concentrations (10 µg/ml) of 405/6 and 147 is as much as four-fold higher for the 405/6 preparation (results not shown). This suggests that the 405/6 preparation, although containing a low affinity component, contains a higher proportion of a high affinity component which dominates the assay response at the low antibody concentrations used in the IRMA titration. Using the approximation of a single antigenic determinant employed above, the comparative ELISA response of the 405/406 and 147 preparations suggests that the 405/406 preparation might contain as much as 8% of the high affinity component. This consistent with the lower <u>limit</u> of 4% set by the IRMA titration.

Although IEF analysis of the anti-H1 preparations shows that the 147 preparation contains a more diverse set of antibodies than the 405/406 preparation, and the cross reaction reaction experiments with Drosophila H1 clearly show that antibodies cross-reacting with Drosophila determinants are almost entirely absent from the 405/406 preparation, this does not exclude the possibility that it may contain more of a more affine species than the 147 preparation. These observations represent examples of variation in the properties

of different polyclonal antibody preparations which were considered under points 1) and 3) in the section R.7.2.4.

Despite the very low binding efficiency of most of the 147 preparation, it is notable that ELISA is still able to detect a signal from an amount of adsorbed H1 which can be no greater than 30 pg H1 (1 femtomole, 6×10^8 molecules) although this signal is clearly very near to the detection limit (cf. R.7.3.1.2.2) as panels b and c in fig. 30 illustrate. The 147 antibody preparation clearly contains a high proportion of low affinity antibody. This suggests that either increasing anti-H1 concentrations or using other antibody preparations containing more affine antibodies might raise still further the signal which could be obtained from such small amounts of H1. Thus the sensitivity limit of the ELISA used here is at least as great, if not greater, than that estimated for IRMA (0.5 femtomole, 3×10^8 molecules).

To summarise, there are 4 lessons to be learned from the analysis and comparison of ELISA and IRMA titrations.

1) The efficiency of antigen binding even by an affinity purified antibody may be extremely low. A 10^4 fold molar excess of antibody over antigen may be required to generate a detectable signal at very low antigen levels. Much of this inefficiency can be traced to a high proportion of low affinity antibodies in affinity purified preparations (see also R.7.3.2.2).

2) The optimal concentration of all components in the assay should be controlled under the most extreme conditions to avoid artefacts such as the apparent saturation of antigen binding by antibody in fig. 30 (in fact limiting detector).

3) Monospecific antibodies can be heterogeneous mixtures.

4) Providing attention is paid to these boring details, subfemtomole sensitivity can be obtained from solid-phase immunoassay.

R.7.3.5 The binding capacity of microtitre plate wells

As fig. 30 in the previous section shows, when a solid phase assay response is titrated with respect to the antigen concentration used to coat the wells, under conditions where detector is non-limiting, the assay response reaches a maximum. This implies that the binding of antigen to the well surface is saturable, presumably because binding occurs by direct attachment of protein to plastic and because the available area of plastic is finite. If the sites to which protein can attach are sufficiently densely distributed one would expect the upper limit to the amount of protein which could be bound to be set by the area exposed to protein and by the maximum packing density allowable to the molecules on such an area. Maximum packing would be determined by the shape and cross-sectional area of the protein molecules in whatever orientation they are bound to the surface, the geometrical arrangement of molecules bound to the surface (regularly arrayed/ randomly oriented) and well surface topology. The microscopic structure of the well surface is unknown. If it were corrugated, or if it were made up of interwoven fibres, and if the spacing between the corrugations or fibres exceeded the dimensions of the antigen to be adsorbed, then the surface area of the well might conceivably be several-fold greater than if the well surface were smooth (or showed deviations from smoothness only at dimensions less than those of antigen molecules).

On the basis of these considerations, and of the dependence of assay response in antigen titrations I suggest a general model to describe binding of antigen to the surface of microtitre plate wells

i) To a first approximation the surface of the wells can be considered smooth.

ii) Initially antigen is transferred quantitatively from solution to the well surface at the diffusion-limited rate.

iii) The amount of antigen which is required to saturate a plate surface is therefore the same for wells made of different materials.

It is however clear from the results reported in section R.7.3.5.2 that although the concentration of antigen required to cause saturation of the assay response is the same for different kinds of plates, the absolute assay response obtained at saturation from plates made of different materials is different. This can be explained as follows:

iv) Immunologically reactive antigen is subsequently lost from plates either physically (i.e. by desorption), or by "inactivation" of antigen either by denaturation or by inaccessibility of antigenic determinants (cf. R.7.2.2.1).

v) Different amounts of antigen loss from plate surfaces made of different materials result in different absolute assay signals, although the same amount of antigen was initially adsorbed.

vi) The same arguments as in iv) and v) above can be used to explain differences in absolute assay values which occur when antigen is adsorbed to plates under different physical conditions (e.g.temperature) by supposing that the inactivation of adsorbed protein differs under the different conditions.

The following two sections outline the detailed evidence for this model.

R.7.3.5.1 Initial transfer of antigen to the well walls is quantitative

In section R.7.3.3 I argued that the transfer of antigen to the well walls took place mainly by a diffusive mechanism, on the assumption that the immunological signal generated by antigen adsorbed to the walls for different lengths of time

was directly proportional to the physical amount of antigen adsorbed to the well walls. Given a maximum assay response generated by a limiting amount of antigen (MX_{lim}), I assumed that the fraction of MX_{lim} observed after different periods of incubation corresponded to the fraction of antigen removed from bulk solution by adsorption.

An objection to this argument is that there might be a binding equilibrium between the antigen and the plate surface, such that, even when antigen was limiting, only a small fraction of the antigen (say 1%) might be physically transferred. If this were the case then the fraction of MX_{lim} observed after different periods of incubation would not be a suitable measure of physical transfer.

The counterargument to this objection is based on the result of a serial transfer experiment, in which an antigen solution was allowed to coat a well for only a short period of time and was then transferred to an untreated well and the process repeated.

If only a small proportion of the limiting amount of antigen present in solution were removed by adsorption, then the concentration of antigen remaining in solution would remain nearly unchanged, and the immunological signal obtained from sequentially coated wells would be expected to remain nearly constant, or to decline less rapidly than theoretically expected. If on the other hand the proportion of antigen removed was equal to the proportion of the maximum immunological response MX_{lim} to which it gave rise, then the immunological signal from the coated wells after each transfer would be expected to decrease by the proportion transferred. For transfers of equal duration this is formally equivalent to a first order decay process, for which the signal (S_n) n transfers after an initial transfer (n=0) will be related to the signal after the initial transfer (N_o) by the expression

$$S_n = N_o e^{-\lambda n}$$

where e = the numerical constant 2.7183.... and λ is the fraction adsorbed/lost from solution at each transfer. A plot of log S_n against n should give a straight line of slope λ and ordinate intercept at n=0 of N_o.

Figure 31 shows that the experimental results obtained from serial transfer conform closely to the result expected if transfer were quantitative. The fraction of immunologically detectable antigen adsorbed at each transfer decreases by 24% (with 95% confidence limits 23% - 25%) compared with a theoretically expected decrease of 18%. Possibly the frequent mechanical agitation of the plates required to transfer antigen every 15 minutes, or adsorption of antigen to the disposable pipette tips used at each could account for the higher than expected rate of antigen adsorption .

These results strongly support the notion that the initial transfer of antigen to the well walls is quantitative.

The constancy of the assay response obtained from the wells incubated with the same amount of antigen after exposure to the same amount of BSA for very different times or to different amounts of BSA for the same time (10^4 concentration range tested, results not shown) argues that adsorbed antigen which is immunologically detectable is essentially irreversibly bound. This would appear to rule out the possibility that there is a reversible equilibrium between antigens and binding sites for **immunologically detectable bound antigen.** However, the data does not exclude the possibility that a different class of binding sites might exist to which antigen might be reversibly bound and lost after initial adsorbtion.

I have not been able to find any data in the literature about the uniformity of binding to PVC for different antigens, but there are reports in the literature about heterogeneous attachment strengths for antigen initially bound to polystyrene. Both CANTARERO et al. (1980) and LEHOTONEN and VILAJEN (1980) observed that washing the plastic several times was necessary to obtain a constant signal from radioactively

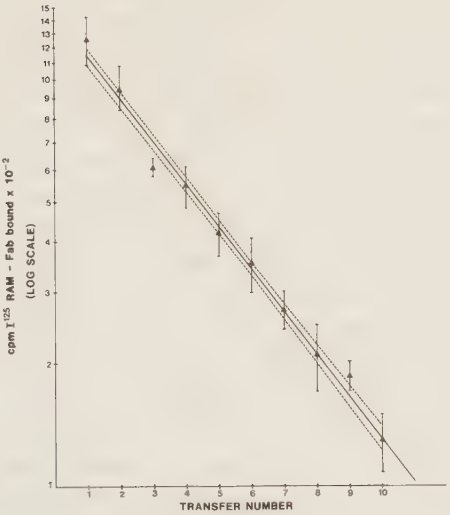

Fig. 31 **The fraction of antigen lost by adsorption to the walls of a microtitre plate well is measureable.**

10 ng Mouse IgG in 100 µl was incubated in a PVC microtitre plate well at room temperature for 15 ± 1 minute and then transferred to a fresh untreated well. To allow for volume losses during transfer (it is difficult to transfer the last 1 or 2 µl from an Eppendorf pipette tip, and such errors would accumulate after 10 transfers) 12 wells were initially incubated, their contents pooled in a vessel which had been precoated with BSA and the contents redistributed to 11 further wells. Pooling and redistribution to a 1 smaller set of wells was repeated at each transfer. After removal of antigen wells were blocked with 0.1% BSA for at least 30 minutes and then processed as usual for the direct detection of mouse IgG. 79400 SE ± 1360 cpm I^{125} RAM Fab, incubated overnight with the adsorbed IgG, was used as detector. Error bars show the standard error associated with each determination. The number of replicate determinations decreases from 12 at transfer 1 to 3 at transfer 10. The solid line shows a linear regression of the logarithm of the bound counts on the transfer number for the original 75 data data points fitted by the method of least squares. The dashed lines indicate the 95% confidence limits of the y on x regression values (M.7.2). Transfer number 1 corresponds to n=0 in the equation above.

labelled protein bound to the plastic surface. CANTARERO et al. (1980), investigated a range of proteins and found that the final proportion of the input labelled protein which

remained stably bound to polystyrene was constant over a wide range of concentrations for any one protein but varied between 10% and 80% between proteins. Furthermore, the extent of retention was roughly correlated with the size of the adsorbed protein. However, the proportion of adsorbed protein fell rapidly when the amount of protein incubated exceeded that required to form a monomolecular film on the plastic surface, suggesting an initial quantitative transfer. The conditions of CANTARERO et al.'s experiments were such that diffusion artefacts can be neglected. In contrast to these examples of inefficient retention, KENNEL (1982) claims that the D fragment of human fibrinogen is both quantitatively adsorbed to and retained on polystyrene. However, even in this case, as with the other antigens, the proportion of the total protein in a well which is adsorbed decreases rapidly as a function of increasing total protein when the total amount of protein is greater than that required to give a monomolecular film (cf. R.7.3.5.2).

Thus, the assumption that all protein reaching the walls of a plastic vessel will be adsorbed until the protein is closely packed on the surface of the plastic appears to be supported by the available data.

R.7.3.5.2 Different operational "binding capacities" of PVC and polystyrene microtitre plates

Table 26 shows the ELISA assay responses obtained from PVC and polystyrene microtitre plates treated under identical conditions. Adsorbtion of antigen to each kind of plate has been examined at two different tempeatures using three different antibody preparations. To avoid any misunderstanding, I should stress that in the experiments in which the effect of temperature on antigen adsorption was studied, that only the adsorption and blocking incubations were made at different temperatures. All subsequent steps were performed at room temperature.

Plate Material	WARM			COLD		
	anti-H1 used			anti-H1 used		
	147	402	405/6	147	402	405/6
	Assay value (E.U)			Assay value (E.U)		
			500 ng H1			
PVC	90	91	94	108	122	120
PS	38	55	77	23	33	60
			50 ng H1			
PVC	25	48	53	40	67	65
PS	14	19	26	11	16	18

Table 26 **Assay response of H1 adsorbed to different kinds of microtiter plates.**

Either saturating (500 ng) or limiting (50 ng) amounts of H1 were adsorbed to the indicated type of microtitre plate well for 21 hours at room temperature (WARM) or on ice in the cold room (COLD). After blocking with BSA at the same temperature used for adsorbtion, the COLD plates were transferred to room temperature and all subsequent steps in the experiment were carried out at room temperature. A 1/50 dilution of the same biotinyl goat anti-rabbit preparation as that used in sections R.7.3.3 and R.7.3.4.2 and a 1/200 dilution of Avidin phosphatase was used as detector. The values for saturating amounts of H1 were only obtained from a single time point.

Control experiments showed that, for the time period for which adsorption was allowed, 21 hours, 500 ng H1 was sufficient to saturate the assay response on both kinds of plate and 50 ng was insufficient, although >95% of the molecules in the antigen solution would have been able to diffuse to the vessel walls in this time period. The amount of initially bound H1 which is required to saturate the well walls must therefore lie in the interval 50 ng to 500 ng.

There are several other conclusions which can be drawn from the data in table 26.

1) The amount of immunologically detectable H1 is greater on PVC plates than on polystyrene plates.

2) The amount of immunologically detectable antigen on PVC plates is greater when antigen is bound to the plates in the cold rather than at room temperature.

3) In contrast to point ii) the assay response from polystyrene plates is greater if the antigen is adsorbed at room temperature rather than in the cold.

When the assay response is titrated with respect to antigen using narrow intervals between antigen concentrations the approach to saturation is clearly seen to be asymptotic (results not shown). However, to a first approximation, an estimate of the minimum amount of antigen required to saturate the assay Ag_s can be given by linear extrapolation of that part of the response curve where the response is linearly dependent upon antigen concentration (assay response less than half maximum) to the maximum assay response level according to the formula:

$$Ag_s = Ag_1 \times S/L$$

where Ag_1 is a limiting amount of antigen giving rise to an assay response of value L and S is the maximum assay response obtainable from a particular kind of plate.

This gives the results in table 27.

There is a most unsatisfactory amount of scatter in this data, and there appear to be some systematic discrepancies such as the high apparent binding of H1 to polystyrene plates in the warm. Nonetheless, all except two of the values fall within 30% of 124 ng, the mean of pooled data. If there is a real difference in the amount of H1 binding to the different kinds of plate at initial saturation then the difference is unlikely to greater than 30% of the plate with higher initial capacity. In the absence of any more precise data 124 ng is the best estimate of initial H1 binding that is available.

	Amount of H1 bound at saturation (nanograms)			Mean
	anti-H1 used			
	147	402	405/6	
PVC - warm	180	95	89	121
PVC - cold	134	91	92	105
PS[1] - warm	135	149	148	144
PS[1] - cold	109	103	167	126
				124

[1] PS = Polystyrene

Table 27 **Estimates of initial binding capacity of microtitre plates.**
Calculated from the data of table 26.

Titration of the ELISA response with respect to IgG on polystyrene plates suggests that the initial capacity of microtitre plate wells for this antigen is about 240 ng. Further properties of IgG in solid-phase assay are described in section R.4.2.

Table 28 summarises the proposed initial capacity of the wells in molecular terms. The areas initially available to bound molecules are consistent with the proposal by CANTERERO et al., 1980), explained at the beginning of section R.7.3.5, that adsorbtion is limited by the area available to the adsorbing molecule. The data is also consistent with the very approximate estimate of BROOME and GILBERT (1978) of a binding capacity of PVC for IgG of $2\ 10^{10}$ molecules per mm^2. There is no need to assume a surface topology for the wells other than a smooth one. The maximum cross-sectional area of an IgG molecule calculated from its Stokes radius and assuming a spherical molecule is 6600 $Å^2$. On the basis of a more realistic geometry (a thick truncated triangle lying on its

Measure of initial Binding capacity	ANTIGEN H1	IgG
Mass (ng)	120	240
Mass (ng)/mm^2	1.7	3.4
Equivalent concentration (M)*	$5.4\ 10^{-8}$	$1.7\ 10^{-8}$
Molecules/mm^2	$4.56\ 10^{10}$	$1.35\ 10^{10}$
Area / molecule (Å^2)	$2.4\ 10^3$	$7.4\ 10^3$
Dimensions of available area (Å x Å)	48 x 48	86 x 86
Retention of immunologically active molecules on PVC	100% (?)	100% (?)
on PS	30%	50%+

* If material adsorbed on surface were dissolved in 100 µl
+ Figure given by CANTERERO et al. (1980)

Table 28 **Binding characteristics of two antigens to microtitre plate well surfaces.**
An area of 74 mm^2 for the surface of a well filled with 100 µl of liquid has been assumed. In calculating areas per molecule, the binding surface is treated as if it were perfectly smooth.

face) and dimensions determined by direct EM observation and other biophysical methods (VALENTINE and GREEN 1967, AREND et al., 1972) might be as little as 5500 Å^2. In either case 7400 Å^2 would almost certainly be sufficient to allow the molecules to pack closely. Estimating the area occupied by a molecule of H1 is more difficult, because of its unusual highly elongated form (DAVISON et al., 1954; HAYDON and PEACOCKE 1968; HARTMAN et al., 1977). This is thought to consist of a central spherical compact region, to which are attached two very thin highly charged tails (HARTMAN et al., 1977). The cross-sectional area computed from the Stokes radius of the molecule, assuming spherical geometry, would be

too great to accomodate in the available area listed in table 28. However if a considerable proportion of the long thin tails were to orient perpendicular to the well surface then the 2350 Å^2 available to each newly adsorbed molecule would certainly be sufficient to accomodate the central portion which has a cross-sectional area of only 1200 Å^2.

Using **shaken** polystyrene microtitre plates KENNEL (1982) estimates the capacity for fragment D of human fibrinogen at 1000 ng per well. The area exposed to protein in a well on a shaken plate is not given, but on the basis of a 50 µl volume of antigen solution, it must have limits between 53 mm^2 (shaking ineffective) and 180 mm^2 (total area of well). This sets limits on the antigen densities observed by KENNEL of between 2.8 10^{10} and 9.5 10^{10} molecules/mm^2. This implies a packing density for this antigen between 2 and 8 times that found for IgG (the two molecules have comparable molecular weights). Comparison with the arguments presented above for IgG suggest that this high packing density could only be achieved if the D fragment were to bind either as an oligomer or, if it is asymmetric, very closely packed with its longer axis perpendicular to the well surface. It is easy to imagine how either of these arrangements might contribute to the "inactivation" (immunological undetectability) of antigen that this author describes (cf. R.7.2.2.1)

In contrast to the calculations above, which only consider proportionate responses at different antigen concentrations the absolute assay response from PS plates can be lower than that from PVC plates by a factor 2 to 4. This need not involve a contradiction. As I explained in the previous section, if the initial transfer of antigen is quantitative and the upper limit to initial adsorbtion is determined by antigen packing density (minor differences could be explained by small differences in surface smoothness or the geometrical arrangement of binding sites), then this could

determine the same proportionate assay response to the antigen concentration when different plate types are used. The absolute response, on the other hand, is a measure of antigen loss/inactivation subsequent to initial binding. Physical loss of adsorbed protein from polystyrene is known to be as high as 90% for some low molecular weight proteins (CANTERERO et al., 1980) and other kinds of inactivation may be equally as great (cf. R.7.2.2.1. and KENNELL 1982). The accessibility of the same antigenic determinants on different kinds of plates might, for instance, be different. Although this is a tempting idea there is little data to support it from the values presented in table 26. The major difference in assay responses between the different anti-H1 preparations is the lower response generated by the 147 preparation, and this can probably be attributed to its lower average affinity.

Differential loss of antigen from different plate types has implications for the absolute sensitivity of ELISA (cf. R.7.3.4.3). Even if the highest assay responses on PVC were to represent the quantitative retention of antigen, examination of table 26 shows that absolute retention of H1 on polystyrene plates is only about 30% of the PVC value meaning that at most this percentage of immunologically reactive H1 is retained on the plates compared to that initially adsorbed. This means that in the ELISA titration whose results are shown in fig 30 at most 1/10 (30% of the 30% diffusion constrained binding) of the amount of H1 to which the wells were initally exposed can have been responsible for generating the assay response. This proportion would be still lower, and the absolute sensitivity of the assay correspondingly higher, if the immunological detectable H1 bound to PVC plates is itself only a fraction of that initially bound.

It is interesting that the initial amount of IgG lost from polystyrene is estimated by CANTERERO to be 50%, because the assay response which can be obtained by binding IgG to PVC

plates is a factor 2 greater. This might mean that not only is IgG quantitatively bound to PVC plates, but also quantitatively retained. The estimate of BROOME and GILBERT (1978) for IgG binding to PVC would also support this idea.

An attractive hypothesis which would allow a unified explanation of the binding data can be built up on the following three assumptions.

i) two different interconvertible classes of sites, weak and strong, on the plastic surface to which protein can attach.

ii) randomly distributed sites of each class at a density such that a body with the dimensions of a protein molecule will come into contact with several such sites.

iii) if more than a certain number of strong attachments occurs the protein will become irreversibly bound.

Such a model could explain 5 of the observed effects.

1) Alterations in temperature could cause conversion of weak sites to strong sites or vice versa depending on the the direction of the temperature change and the type of plastic.
2) Binding even to only weak sites or to a subcritical number of strong sites is sufficiently strong to allow initial attachment, explaining the quantitative removal of proteins from coating solutions.
3) Physical loss of initially bound protein can be explained by presuming that proteins bound only to weak sites, or to a subcritical number of strong sites, will be removed by subsequent washing or competition by the BSA used to block free binding capacity on the wells.
4) The rough correlation between molecular weight and the efficiency of retention can be simply explained by presuming that the contact area of the protein will determine the probability that the critical number of strong sites will be met.
5) The greater retention of protein on PVC plates than on

polystyrene plates could be explained either by presuming a greater proportion of strong sites on the PVC plate, a different density of sites, or a different critical number of strong attachment sites for irreversible binding. Combinations of these effects would also be possible.

Such a model is potentially testable, although it would first be necessary to cast it in more precise mathematical form to allow the retention behaviour as a function of molecular size and shape and of the various optional assumptions to be calculated. Once such predictions were available they could be compared with the real behaviour of a series of different proteins on different kinds of plastic.

In summary, it appears possible to estimate the binding capacity of microtitre plate well surfaces for purified proteins. The upper limit appears to be set by the number of molecules that can pack into a monomolecular film, but may be less than this (but probably not less than 10% of the theoretical limit). For a molecule intermediate in size between H1 and IgG a monomolecular film would contain approximately $3\ 10^{10}$ molecules mm^{-2}. Given an approximate area of 1 cm^2 exposed to antigen in a microtitre plate well this indicates a maximum capacity **per well** of approximately $3\ 10^{12}$ molecules. Given a lower detection limit of approximately $3\ 10^{8}$ adsorbed molecules per well (R.7.3.4), this suggests that any species in a protein mixture must make up at least 0.01% of the molecules adsorbed from that mixture in order to be detectable. However, as I show in the next chapter (R.7.4), predicting the proportion of molecules adsorbed from mixtures presents formidable problems.

R.7.4. Solid phase assay of antigen mixtures and binding characteristics of monoclonal antibodies

The properties of solid phase assay which I have outlined in the previous parts of this chapter allow some predictions of the behaviour of antigens in mixtures, such as crude preparations of Drosophila nuclei. The effects will vary depending on the total amount of adsorbable material in the mixture. If this amount is insufficient to cause saturation of the well surface, non-interacting antigens would be expected to behave essentially independently of one another (cf. also R.8). If material in the mixture is present in excess of the binding capacity of the well surface, competition between different components would be expected. If the mixture only contains soluble proteins, the proportion of the total adsorbed protein made up by a particular component would be expected, a priori, to be a function not only of its relative concentration in the mixture but also by its relative rate of diffusion to the vessel walls.

If the aim of using solid phase assay of an antigen-containing mixture is to obtain information about the relative or absolute amounts of an antigen in the mixture then at least five problems arise. Three of these are common to assays in which either saturating or unsaturating amounts of adsorbed antigen are used. The remaining problems concern the efficiency of adsorbtion and will depend upon whether the the well surface has been saturated by the mixture.

The first of the common problems is that, when indirect technique is used, it will only be possible to determine the absolute amounts of a bound monoclonal antibody (and hence adsorbed antigen) if the detector signal can be calibrated in molecular amounts. This requires a knowledge of the stoichiometry of detector:monoclonal binding and of the binding characteristics of the detector. Estimates of these properties are reported in sections R.7.4.5 and R.7.4.6.

Secondly, some knowledge of the extent of antigen inactivation (if any) is required. Even to determine the ratio

between two antigens the relative inactivation of each would have to be known. Satisfying this requirement would probably require an independent assay system.

Thirdly, the efficiency of the monoclonal antibody binding reaction must be known: this requires an estimate of antibody affinity. To a first approximation, this should be possible by simply titrating the assay response with respect to antibody (R.7.4.4, R.7.4.4.2, R.7.4.5). Under certain conditions, if the assay response saturates, the reciprocal of the antibody concentration required to give a half-maximal assay response will be equal to K_{eq}. The ability to carry out titrations of this sort gives information about the upper limit of the amount of adsorbed antigen in the system. However, if the affinity of the antibody is low and non-specific binding effects (R.7.3.1.2.3, R.7.4.4.1) prevent an unambiguous determination of the assay signal at saturation, it will not be possible to use this procedure

Where the amount of a mixture is not sufficient to saturate the adsorbtion capacity of the well surface, account has to be taken of the time allowed for adsorption. The adsorbed proportion of individual antigens would vary according to their diffusion coefficients. For comparison of low M.W. antigens ($2\ 10^4$ - 10^5 daltons) with similar diffusion coefficients ($D_{20,w}$, approx $5\ 10^{-7}$ $cm^2\ s^{-1}$) this effect could be neglected, and for larger soluble proteins with M.W. > $2\ 10^5$ ($D_{20,w} < 2\ 10^{-7}$ $cm^2\ s^{-1}$) differences in relative assay signal as a result of differential transport would be minimised if long periods of adsorbtion (18 hr, proportion adsorbed > 80%) were used. However, if an antigen were part of a large particle (>10S), with a low diffusion coefficient ($D_{20,w} < 10^{-8}$ $cm^2\ s^{-1}$) then after 18 hr only 24% of the particles and even after 24 hours **at most** 28% of the particles would be adsorbed. Thus, artefactual differences of a factor 3 could easily be generated. If the native size of two antigens were unknown, gross differences could be tested for, by

comparing the rate of disappearance of antigen using the serial transfer technique (cf. R.7.3.5.1 and R.7.4.3). Probably, if the particles are massive enough, sedimentation to the bottom surface of the well would tend to compensate for very low rates of diffusion, but this would only become important for very massive particles (M.W. >> 5 10^7 Daltons, S >> 50S, at which mass the energy to drive directed transport caused by the gravitational potential becomes greater than the random motion resulting from thermal effects).

In the case that adsorbable material is able to saturate the plate, there will be competition between antigens for the limited surface area available. Especially for complex mixtures, the precise outcome of this competition is difficult, if not impossible, to predict. I have not determined exactly what the outcome of the dependence on the two a priori variables (relative abundance and relative rates of diffusion) for any particular species in a mixture will be. However it is clear, from empirical determinations (R.7.4.1), that there are concentration dependent effects. It difficult to determine whether or not other mechanisms such as the direct displacement or excluded volume effects (R.7.2.2.2) may also be operative.

If competition is suspected to be depressing the assay response of an antigen because it is part of a very slowly diffusing particle, this can be tested empirically, by seeing if it is possible obtain an enhanced assay response under conditions where a particle might be expected to break up into its (much more rapidly diffusing) constituent units. Another empirical test for disproportionate depression of an assay response to a particular antigen as a result of competition is to dilute the antigen mixture and see if the assay response rises as the total amount of antigen moves into the range where antigens behave independently (adsorption non-saturating see above). Such effects can be demonstrated (see R.7.4.1).

Thus, even if it possible to measure the amount of

adsorbed antigen precisely, a major problem remains in attempting to relate this quantity to the frequency of antigen in the original mixture. In the absence of any antigens which can be used as standards (i.e. whose true proportion in the mixture and whose diffusion characteristics are known) it is not possible to know whether there are any general rules (even empirical ones) which can be deduced concerning this relationship. This uncertainty, rather than any other factor, makes it difficult to judge the reliability of estimates of antigen frequency in Drosophila cell nuclei, which have been obtained by solid phase assay methods (see R.9.2.2).

R.7.4.1 Competition between H1 and other proteins for adsorption to solid phase

The results in this and the next section document some ways in which the assay response to the antigen histone 1 is altered by the presence of other proteins in the solution from which it is adsorbed to the solid phase. Although the data are fragmentary, rather than systematic, there are three major conclusions to which they point. Firstly, as expected, the amount of an antigen which is adsorbed is reduced by the presence of other proteins. Secondly, the amount of protein which is adsorbed is not in any simple way proportional to the amount of antigen in the mixture. Thirdly, in crude extracts of nuclei (R.7.4.2), the amount of an antigen which is adsorbed may be highly dependent upon ionic strength.

The effect of other proteins on the adsorbtion of H1 was investigated by using a commercially available, H1 containing, (total) "histone" extract of calf thymus to coat the wells. The concentration both of total histone used to coat the wells and of anti-H1 antibody was titrated. The assay was carried out in parallel to a similar double titration whose results are shown in fig. 28. The response surface of the double titration of the histone mixture, shown in fig. 32, has three features distinguishing it from that obtained using pure H1.

1) The amount of H1 which is immunologically detected is strongly dependent upon the concentration of the mixture used to coat the wells. This dependence is not linear: there is a optimum concentration at which a maximum assay response is obtained. Below the optimum, the sub-maximal response can be explained by supposing that the amount of H1 able to coat the well surface is limiting; the total amount of protein in the well at the lowest histone concentration used is certainly insufficient to saturate the well surface. At supra-optimal concentrations, although the amount of H1 in the well must be **greater** than when the optimum concentration is used, the amount of immunologically detectable H1 adsorbed to the wells is **less.**

2) The absolute level of the response is less than that obtained using pure H1 (compare fig. 28). There is some uncertainty regarding the actual amount of H1 present in the mixture (see below) but, because of the form of the assay response surface to pure H1, this conclusion is not affected by the uncertainty. When high concentrations of the mixture are used to coat the well, the assay signal is only about 10% of that obtained using 10 ng pure H1 to coat the wells (=3ng adsorbed). Even the maximal response is only approximately 50% of that obtained using 10 ng to coat the wells. Assuming that the assay signal reflects the amount of H1 actually adsorbed to the well surface (i.e. excluding such possibilities as reduced accesssibility of adsorbed H1), it suggests that the absolute amount of H1 adsorbed from the mixture at the highest concentrations is less than 1% of that which would be adsorbed from a solution containing a comparable amount of H1 in the absence of other proteins. At the optimum concentration, at which the logical maximum of H1 which could adsorb was 30 ng, the signal from the mixture was less than that produced by the adsorbtion of 3 ng pure H1. This suggests that, even at the concentration most favourable for H1 adsorption, for the core histones cause the absolute amount of adsorbed H1 to be depressed by at least 90%.

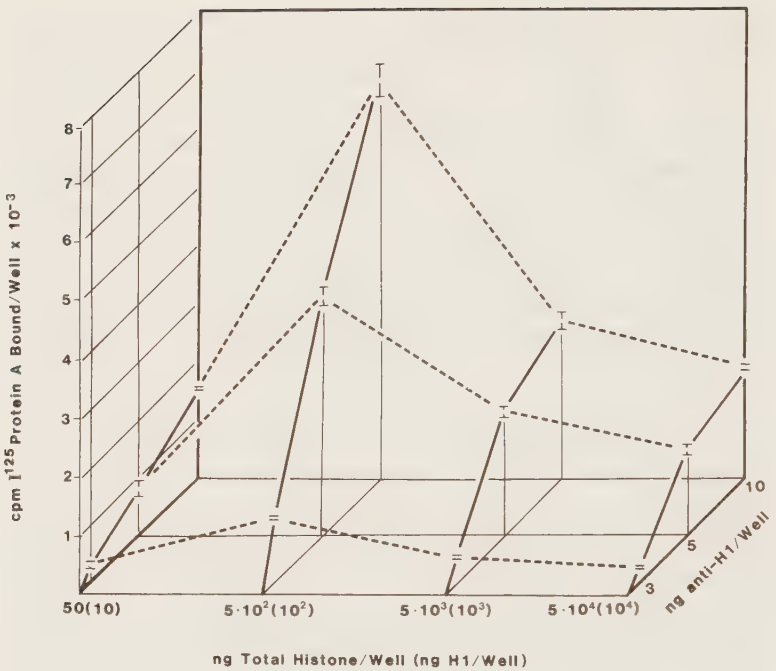

Fig. 32 **Response surface of ^{125}I Protein A binding in IRMA of H1 in a mixture of total histones titrated with respect to both antigen and (first) antibody**

Conditions were exactly as described in the legend to fig. 28, except that the wells were incubated with the indicated amounts of a mixture of total calf thymus histones (Boehringer) instead of pure H1. The abscissa scale refers to the total amount of protein placed in the well, **not** the amount of protein adsorbed. The amounts of H1 indicated by the numbers in brackets on abscissa scale are nominal and may be in error by at most a factor 4 (too high, see text).

3) At the two highest concentrations of histone mixture used, the assay response as a function of anti-H1 appears to saturate. This is in contrast to the results obtained using pure H1, where the assay response was (to a first approximation) directly proportional to the amount of anti-H1 used over the whole range tested. This finding is in keeping with observation 2) above and suggests that in comparison to the amount of H1 which would be adsorbed from a solution of an

equivalent concentration of pure H1 only a very small amount (<1%) is adsorbed from the mixture. At the optimum concentration of the histone mixture, the assay response appears to be more nearly proportional to the amount of anti-H1 used in the assay, suggesting that anti-H1 is still limiting. This implies that, if still higher concentrations of anti-H1 had been used, the peak in the assay response surface would have been still more pronounced.

The simplest explanation of these features is that the depressed assay response at high concentrations of the mixture represents lower amounts of adsorbed H1 (as opposed, for instance, to a reduction in the binding of anti-H1), and that the reduction in the amount of adsorption is caused by the occupation of potential binding sites by the other histone molecules. Unfortunately, the exact amount of immunologically active H1 in the mixture used is not known. Nominally, a total histone extract should contain 20% by weight H1 (approximately 10% on a molar basis). The amounts of mixture used to coat the wells were chosen on this basis, to make the concentrations of H1 used for the titration equivalent to those used for the titration shown in fig. 28. However, subsequent SDS-PAGE analysis of the histone mixture showed that, in addition to intact H1 (identity confirmed by a very strong autoradiographic signal in Western blotting, results not shown), it contained a number of polypeptides with molecular weights intermediate to those of H1 and the core histones, which were presumably H1 degradation products. No attempt was made to estimate the extent of degradation exactly, but the nominal amount of H1 could certainly not have been in error by more than a factor 4. Although this uncertainty is annoying, it does not qualitatively affect the major conclusion to be drawn from the observations made above: namely that, at high mixture concentrations, the other histones cause an reduction in the adsorbtion of H1 more than an order of magnitude **greater** than that expected if each species of protein were to bind in

proportion to its molecular frequency. At the optimum mixture concentration for the H1 assay response, this ratio is clearly more nearly approached.

What is most disturbing about these results is that the concentration dependence of the H1 assay response for H1 in mixtures is not what one would intuitively (albeit naively) expect. This behaviour does not appear to be limited to H1 in simple mixtures. A similar effect appears to be operative when crude nuclear preparations are assayed for H1 (see below). The binding of mouse IgG to plastic also shows a concentration dependent optimum (see R.8) suggesting that its binding may be similarly affected (i.e. by exclusion from adsorption) by competing proteins. The mechanism of exclusion is not known, but one can imagine some possibilities: either the excluded antigen reaches the surface but is displaced before it can become irreversibly adsorbed or, alternatively, the amount adsorbed is proportional to the rate of transport to the well surface of each component in the mixture. However, in the latter case, in mixtures, the transport behaviour of each component does not appear to be simply described by its diffusion constant (see R.8.2).

R.7.4.2 The effect of salt on the immunological detectability of H1 in Drosophila Kc cell nuclei

The immunological signal generated by a component in a complex mixture is not only dependent on the concentration of the mixture used to coat the well walls. Changing the ionic strength of such mixtures can also cause large changes in the amount of immunologically detectable antigen. This is illustrated by comparison of the results shown in fig. 33a and 33b. The same batch of Drosophila nuclei was assayed to produce the results shown in each of the panels, but the nuclei used to produce the results shown in fig. 33b were suspended in 2M NaCl before sonication. This treatment results in both quantitative and qualitative changes in the H1 assay

response. The major quantitative change is that the 2.0 M NaCl treatment increases the assay response to H1 by a factor of between 5 and 10 fold with respect to the amount detected when the nuclei are made up in PBS. The major qualitative change is that after salt treatment the signal generated using a nominal 10 µg of nuclear protein is no longer greater than that obtained using 1 µg nuclear protein.

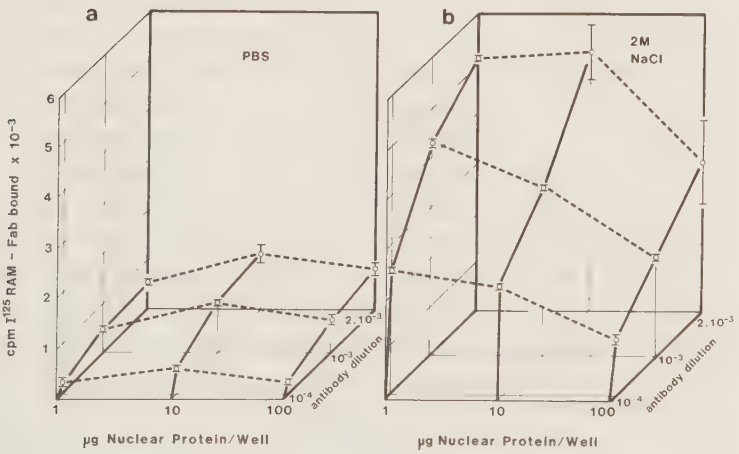

Fig. 33 **^{125}I RAM-Fab binding in IRMA of Drosophila H1 in sonicated Kc nuclei titrated with respect to antigen and antibody**

Conditions of assay were as described in M.2.5.5.4. H1 was immunologically complexed using a monospecific antiserum from mouse #27, (kindly provided by Dr. H.SAUMWEBER) (see text), diluted as indicated on the axis extending into the plane of the paper.

Panel a: nuclei prepared by dilution and sonication in PBS.

Panel b: nuclei prepared by sonication in PBS to which 2M NaCl had been added.

Means of duplicate determinations are shown: error bars indicate the range. Input of ^{125}I RAM-Fab 7.16 $\pm$ 0.12 (95% confidence limits) 10^4 cpm. Results have been corrected for binding of mouse antiserum to BSA (47, 97 or 186 cpm at 10^{-4}, 10^{-3} and $2\ 10^{-3}$ dilutions respectively, in PBS: 85, 389 and 418 cpm in 2M NaCl) and for binding of RAM-Fab directly to nuclei (56 cpm and 23 cpm to 100 µg PBS and 2M NaCl nuclei respectively).

For the nuclei made up in PBS, there appears to be a weakly expressed maximum assay response using 10 µg of nuclear protein per well. It is not possible to make any definite conclusions about the concentration of 2M NaCl nuclei required to give a maximal H1 assay signal, because only 3 concentrations and order of magnitude steps have been used, but it is certainly lower than that required for the PBS nuclei.

It seems unlikely that the increase in assay response was caused by a change in the intrinsic immunological reactivity of H1 itself. In section R.7.3.4.1.1 I have presented results which show that salt does not appear to increase either the equilibrium position of the H1-anti H1 reaction, for at least one anti-H1 preparation, or the intrinsic capacity of the well surface for binding protein. The effect of 2M NaCl, during the adsorption stage, upon the assay response of pure H1 was, if anything, to lower it. It is more likely that the effect of 2M NaCl is to alter the interaction of H1 with other components in the nuclear preparation. Unfortunately, the antibody used to obtain the results shown in fig. 29 did not crossreact with Drosophila H1 (cf. R.11.1), so it is only possible to argue by analogy that the conclusions drawn from the results presented in section R.7.3.4.1.1 also hold for the system used to obtain the results shown in fig. 33.

The results shown in fig. 33 were obtained using a monospecific mouse antiserum raised using a histone enriched Drosophila nuclear protein fraction as antigen. This antiserum was found by Western blotting (results not shown), to react with only a single polypeptide in Drosophila nuclei, which migrated to exactly the same position as Drosophila H1. It was used because, at the time these experiments were performed, it was not known that the rabbit antibodies also cross reacted with Drosophila H1. Since the reactive antibody in this antisera had not been purified by affinity chromatography, it would be formally possible to argue that some other component of the Drosophila nuclei than H1 was being detected by this

antiserum. I would prefer to believe that this was not the case, especially since the assay behaviour of crossreacting rabbit anti-H1 in response to 2M NaCl was very similar to that found for the mouse antiserum (see below). However, even if the antigen whose response is shown in fig. 33 were not H1, this would not alter the conclusion that there are antigenic components of the Drosophila nucleus whose apparent titre in solid phase assay can be drastically altered by changes in ionic strength.

It would appear that the assay response of other antigens in the Drosophila nucleus is not affected by ionic strength in the same way as that for H1. Table 29 shows the relative assay response obtained using either anti-H1 or anti-core histone antibodies against Drosophila nuclei under two different sets of conditions. For technical reasons, a smaller area of the plate well surface was coated using 50 µg nuclear protein per well than 5 µg, so that in terms of the absolute response the results are not directly comparable. However, it is legitimate to compare the ratio between the responses under the two different conditions. This comparison (rightmost column of figures in the table) shows that, for the core histones, the assay response to the low salt, high antigen concentration combination is four fold higher than that to a combination of high salt and low antigen concentration, despite the smaller well area covered by antigen at the higher antigen concentration. Furthermore, the ratio is the same for each of the core histones, suggesting that they all behave similarly. H1 shows the contrary behaviour, the assay response to H1 at high salt is greater, despite the lesser amount of nuclei used in the wells, but in agreement with the behaviour shown in fig. 33. Even correcting for the difference in surface area used for adsorbtion (approximately a 30% increase in the 50 µg values would be expected), it is clear that the ratio of the immunological signals between H1 and any of the core histones (n.b. another ratio, not that referred to in table 29), can be

altered by as much as a factor 10 by manipulating the conditions under which antigen adsorption takes place.

Antibody	Assay response (cpm ^{125}I Protein A bound) Antigen Source		Response ratio
	2M Nuclei 5 µg/well	PBS Nuclei 50 µg/well	PBS(50µg):2M(5µg)
anti-H1	9763 ± 150	2541 ± 287	0.26
anti-H4	1032 ± 208	4395 ± 288	4.2
anti-H3+H2A	658 ± 342	2649 ± 411	4.0
anti-H2B	3337 ± 600	13007 ± 1724	3.9

Table 29 **Effect of salt and antigen concentration on IRMA response to H1 and core histones**

^{125}I Protein A binding to the antibodies indicated at the left of the table bound to antigens from Drosophila Kc cell nuclei treated as indicated at the head of the second and third column of figures. PVC microtitre plates were used and coated with either 50 µg or 5 µg nuclei sonicated, respectively in either PBS, or PBS to which 2M NaCl had been added. Monospecific rabbit anti-core histone antisera were kindly provided by H. WILL (Heidelberg) and were each used at a dilution of 1:800. The anti-H1 antibody was from rabbit 402 (cf. R.4.1) and was used at a concentration of 2.6 µg/ml. All values have been corrected for binding of antiserum to BSA (varied from serum to serum between 300 and 900 cpm) and binding of ^{125}I protein A to nuclei (130 cpm (PBS), 150 cpm (2M)). Error values indicate either range of duplicates (50 µg PBS values) or 95% confidence limits for quadruplicate determinations (2M NaCl, 5 µg nuclei). Input of detector was 83,000 ± 5,700 (95% confidence limits) cpm.

An explanation of the 2M NaCl effect on the apparent titre of H1 may be easily found. In PBS most of the H1 in a nuclear sonicate will be present in very high molecular weight, very slowly diffusing, chromatin structures. In contrast, at 2M salt, H1 will exist in the form of free molecules: H1 is known to be stripped from chromatin at salt concentrations greater than 700 mM NaCl (OHLENBUSCH et al., 1967). The increased diffusive flux of the free molecules to the vessel walls could then account for its enhanced titre. In contrast, the core histones are only partially stripped

from chromatin at 2M NaCl and remain in oligomeric form (PHILIP et al., 1979). In qualitative agreement with such an explanation, the solid phase assay titre of the hnRNP associated Drosophila antigens S5-ag and T7-ag which are known to form part of salt sensitive particles (RISAU 1983), also increases by a factor of 2 when 2M NaCl rather than PBS treated nuclear sonicates are used as a source of antigen (results not shown).

Although the explanation for the salt effects is simple, the implications are far reaching. In preparations of nuclei sonicated in PBS, the monoclonal antibody defined antigens investigated in this thesis are mostly in particulate form (RISAU et al., 1983). These antigens have different distributions in sucrose density gradients following centrifugation of nuclear extracts and the sensitivity of the distribution of each of the antigens to salt is known to vary greatly between different antigens (see fig. 21 in RISAU 1983). The breadth and variablilty of the sedimentation velocity distributions which are found (e.g. P11-ag) could be explained in part if the binding of the antigens in particles were sufficiently weak that an equilibrium between free and bound forms of antigen existed whose position could be altered by ionic strength. This does not of course mean that the antigens cannot also be considered to be part of particles whose size varies with ionic strength.

Given antigens with such properties, if the explanation for the effect of salt on the on H1 assay response is correct, it follows that the amount of many adsorbed antigens is unlikely to directly mirror their frequency in the nucleus. Rather, adsorbtion ratios will probably be a complicated function of the distribution of antigen between free and particulate forms or between particles of different sizes. Particulate antigens are likely to be inefficiently detected (but see next section), whereas "soluble" antigens are likely to be overproportionately represented in the adsorbed

population, with respect to their true frequency in the nucleus.

Thus, without any additional information about the native form of the antigen, it would seem unwise to use the magnitude of a solid phase assay response as an indicator of an antigen's frequency, especially if the antigen source is known to contain particulate material. The examples in table 29 indicate that this mechanism could result in estimates of relative antigen frequency which are in error by a factor of at least 10. Given additional sources of variation between antibodies at subsequent stages of the assay (see following sections of this chapter), it would seem quite possible that antigens with identical molar frequencies might generate assay signals varying by two orders of magnitude.

This does not mean that solid phase assay is useless. As I pointed out at the beginning of this chapter (p. 193), it is an empirical method, chosen because it gives reproducible results. It provides an (albeit arbitrary) way of screening hybridomas for specific antibody production and allows specific antibody production to be followed during subsequent cloning steps. It has served well in this respect. Indeed, because of its convenience solid phase assay will probably continue to be the method of choice for monitoring the progress of antibody production (see also R.8). Under certain conditions solid phase assay can also give useful quantitative information (see R.9.2). However, solid phase assays, using crude nuclear preparations as a source of an antigen, are clearly unsuitable for selecting antibodies against antigens occurring with particular frequencies.

R.7.4.3 The capacity of PVC microtitre plates for sonicated Drosophila Kc cell nuclear protein

In section R.7.3.5, I gave estimates of the binding capacity of the microtitre plate well surface for pure soluble

proteins. As I indicated in the previous section, it is also possible to use sonicated Kc nuclei as a source of antigen. However, the suspension which is obtained by sonicating nuclei contains a large amount of particulate material (see SAUMWEBER et al., 1980 and R.9.1). Some of this particulate material must be very large: not only is the suspension highly light scattering (turbid), but some of it settles out on standing. This raises the question of what the capacity of a plate well surface for such heterogeneous material might be.

I have only attempted to measure the binding capacity of PVC plates directly but, judging by the detectability of antigens in ELISA when crude nuclear preparations are used as an antigen source, it will not be greatly different in polystyrene plates. The best estimate that I am able to provide is that the retention capacity of a plate well surface covered by 100 µl of a nuclear sonicate is

858 ng,

but this may be an underestimate by as much as a factor 2.

The estimate is based on the retention of ^{35}S methionine labelled nuclear protein and upon titration of the IRMA response with different amounts of Kc nuclear sonicate. Table 30 shows the data for the ^{35}S methionine labelled nuclei. Approximately 20% of the material added to the well is initially adsorbed, but only half of this material is retained after the "blocking" step with BSA (final column in table). Thereafter the amount retained remains approximately constant through a further 6 changes of solution, indicating the practically irreversible nature of adsorption (see also R.7.3.3 and R.7.3.5.1), until the final incubation with detector, during which a further 10% to 20% of the the adsorbed material appears to be lost. This final loss is not suprising, because the detector solution contains both salt (1M NaCl) and detergent. It easy to imagine that these harsher conditions might lead to the removal of a fraction of the

IRMA Step	cpm bound/ well	% sonicated nuclei	% initially adsorbed protein
(Sonicated nuclei	25077 ± 516	100	n.a*)
Initially adsorbed	4455 ± 506	17	100
After BSA block	2323 ± 394	9	52
After wash 1	2183 ± 179	9	49
After wash 2	2208 ± 249	9	50
After incubation with monoclonal antibody	2180 ± 307	9	49
After postmonoclonal wash 1	2039 ± 143	8	46
After postmonoclonal wash 2	2029 ± 113	8	46
After mock incubation with radiolabelled detector	1581 ± 196	6	36
After postdetector wash 1	1596 ± 242	6	36
After postdetector wash 2 (stage at which IRMA is usually evaluated)	1862 ± 148	7	42
Chosen retention estimate	1729 ± 140	7	39

* n.a. not applicable

Table 30 **Retention of ^{35}S methionine labelled Drosophila Kc nuclear protein by PVC microtitre plate well surfaces during IRMA**

100 µl sonicated Drosophila Kc cell nuclei, containing 6.6 µg protein (Bradford Assay) and the activity shown, was incubated on ice for 1 hour in each of the 96 wells of a PVC microtitre plate. After each step in the assay, a strip of 8 wells was separated from the remainder of the plate, dried, and counted by liquid scintillation. To avoid the complication of double labelling, radiolabelled antibody was replaced by an equal amount of unlabelled antibody, but otherwise this incubation was identical to that made using radioactive detector. Indicated errors are 95% confidence limits.

adsorbed material or to the dissociation of adsorbed particles. There is a discrepancy in the data in that the amount of material retained in the wells after the final wash appears to be greater than the amount retained after the previous two washes. This is probably simply an unfortunate result of statistical fluctuation which, as the confidence limits indicate,were rather high. In view of this variation I have chosen the mean of the final two steps, which is equivalent to 455 ng protein, with confidence limits 409 ng - 502 ng as the best estimate of the final amount of protein adsorbed.

After only 1 hr incubation in the cold, the 6.6 µg protein used to obtain the data shown in table 30 may not have been sufficient to saturate the binding capacity of the well. This is indicated by the results presented in fig. 34a. The upper curve in this figure shows the IRMA assay response using the S5 antibody plotted against the amount of Kc nuclear protein placed in the well during the antigen adsorption step. It is clear from this data that the assay response is an asymptotic function of the amount of Kc placed in the well and that maximum adsorbtion of the S5 antigen has not been reached using even 40 µg nuclear protein. To estimate the maximum S5 assay response using an unlimited amount of protein to adsorb to the well, a double reciprocal plot of the the data presented in fig. 34a was made, and is shown in fig. 34b. The linear fit to this data is extremely good, and indicates that the maximum S5 assay response (presumably when the well surface is saturated with protein) will be 8690 cpm, with 95% confidence limits 8060 - 9520 cpm. The assay response obtained using 6.6 µg protein to coat the wells was 4600 cpm, or 53% of the maximum signal. Thus, on the assumption that the assay signal is proportional to the amount of adsorbed protein, and that the **adsorbtion** of S5 antigen parallels the adsorbtion of total protein, it possible to calculate that the maximum capacity of the wells for total protein will be 858 ng (455 ng x 100/53), with worst case limits 720 ng to 1040 ng.

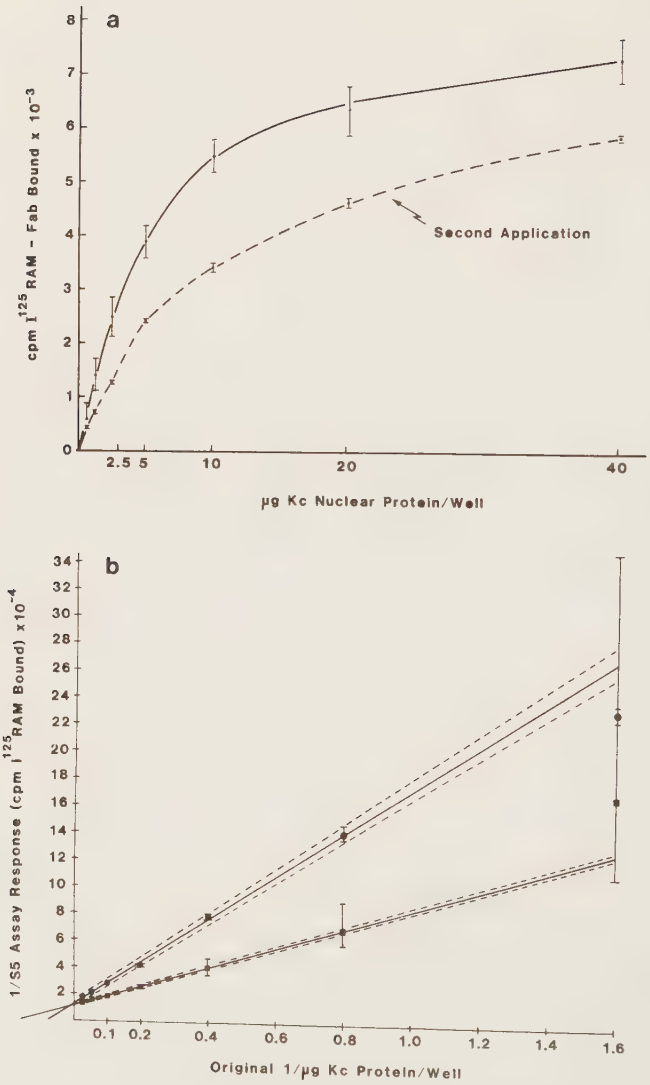
a
cpm I^{125} RAM - Fab Bound x 10^{-3}
Second Application
µg Kc Nuclear Protein/Well
b
1/S5 Assay Response (cpm I^{125} RAM Bound) x10^{-4}
Original 1/µg Kc Protein/Well

Fig. 34 **Assay response of IRMA using the S5 monoclonal antibody as a function of the amount of nuclear sonicate used as antigen source**

(for legend see next page)

Despite the uncertainty of the correctness of the assumptions in this calculation which follow from the observations made in the previous two sections, this figure is plausible. It is only twice the figure obtained by direct measurement and, assuming a protein density of 1.2 g/cc, would correspond to a layer of protein with an average thickness of about 9.7 nm (limits 8.2 - 11.0). The average thickness of RNP and chromatin fibrils is about 30 nm, which means that about 30% of the surface of the well (approximately the area of the bottom surface of the well) would be covered by such structures at saturation.

There are three caveats to the capacity estimate. Firstly, the composition of the ^{35}S-methionine labelled nuclei was not normal, probably as a result of starvation during labelling, and contained only about 10% of the normal P11-ag content (see R.7.4.5). This might have influenced the amount of protein adsorption from these nuclei.

- -

(Fig. 34, continued from previous page)

100 µl of a suspension of sonicated Drosophila Kc cell nuclei containing the amount of protein indicated on the abscissa was incubated in the microtitre wells for 1 hour on ice. IRMA assay was than carried out as described in section M.2.5.2.4 using 1 µg/ml purified S5/7/7/6 antibody. 1.25 $\pm$ 0.02 (95% confidence limits) 10^5 cpm ^{125}I RAM Fab was used as detector.

Panel a: upper curve indicates the amount of ^{125}I RAM-Fab bound to wells in which antigen had allowed to adsorb from the amount of total nuclear protein indicated on the abscissa. The curve labelled "second application", indicates the amount of ^{125} RAM-Fab bound when the suspensions used to obtain the upper curve were transferred to a second set of wells and antigen allowed to adsorb for a second time. Since the exact amount of protein in the suspensions used to provide the "second application" curve is unknown, they have been plotted against the amount of protein present in the original suspension.

Panel b: double reciprocal plot of the data in panel a. The regressions have been calculated, omitting the rightmost data point, by the method of least squares. Lower line (filled squares), original suspension. Upper line (filled squares), second application. The dashed lines indicate the 95% confidence limits of the y on x regression.

Data points are the means of quintuplicate ("original") or quadruplicate ("second application") determinations. Error bars are 95% confidence limits.

Secondly, the binding of the S5 probably represents some form of selective binding by this antigen. The curve labelled "second application" in fig. 34a, indicates the titres of antigen S5 which are obtained when the Kc nuclear suspensions used to obtain the upper curve in the figure are transferred to a fresh set of wells. At all concentrations of nuclear protein, the assay response is approximately 45% that initially obtained (this is perhaps more clearly seen from the double reciprocal plot in fig. 34b: the "second application" values are also fitted very well by a linear regression, indicating that the form of the binding curve is essentially the same in each case, but the ratio of the slopes original:"second application" is 1:2.2, indicating that if the values on the abscissa for the "second application" regression were corrected by a factor 2.2 this regression would be exactly superimposable on the "original" regression; the reciprocal of 2.2 is 0.45). This indicates that 55% of the S5 containing material in the nuclear suspension has been removed during the initial incubation at all concentrations of the suspension. It does not mean that 55% of the S5 antigen remained adsorbed to the well walls: the asymptotic assay response curves in fig. 34 show that this was patently not the case. If the adsorbable S5-antigen were present in the form of free molecules, and these were approximately spherical, giving them a diffusion coefficient of about $5\ 10^{-7}$ $cm^2\ s^{-1}$ at 4^oC, it is just possible that this proportion of the total S5 would be able to diffuse to the well walls in the period of time available for adsorption. However, direct measurement showed that at the higher concentrations of nuclear protein suspension (40 µg/100 µl), the amount of bulk protein removed by adsorption was not measureable. Thus the proportion of total protein removed from solution during the adsorbtion step was much less than the proportion of S5, indicating some kind of selective process. This preferential removal of S5-ag from the suspension raises the question of whether it reasonable

to assume that the ratio of **adsorbed** S5 to total protein adsorbed was constant over the range tested.

Lastly, it is necessary to remember that the "µg" protein referred to throughout this section really refers to "µg BSA equivalents" measured by the Bradford method. As I documented in section M.1.1.1.1, table 1 (p.10) this measure does not necessarily give a true measure of the absolute amount of protein in a sample. This is also true for sonicated nuclear suspensions. Estimates of protein in these suspensions by the Biuret method gave values 2 fold higher than those estimated by the Bradford method (data not shown). This would suggest that in absolute terms the estimate made using the ^{35}S-methionine labelled nuclei is, in any case, likely to be an underestimate.

In conclusion, in view of the uncertainties associated with the adsorption measurements, while it reasonable to take the value of approximately 1 µg as the best available estimate of the amount of adsorbed nuclear protein, it is possible that this estimate could be in error by as much as a factor 2.

R.7.4.4 Binding of monoclonal antibodies: estimation and ranking of affinities

A prerequisite for being able to use solid phase assay for the detection of antigen is that the binding of antibody to antigen must be specific. i.e. it must be discriminatory and of sufficiently high affinity to be useful. In addition, if any attempt is to be made to quantitate antigen by means of these immunoassays, it must be possible to estimate the maximum amount of the monoclonal antibody which can bind to antigen (i.e. saturation of antigen by antibody). I show in this section that there is only a limited range of antibody concentrations for which these conditions can be fulfilled.

The maximum amount of antibody binding is best estimated

by using one of the linear transforms of the binding equation Eq.(1) discussed on p.200. I have chosen to use the double reciprocal plot which is often referred to as the "Lineweaver-Burke" plot to obtain such estimates. The equation describing this plot, using the notation of Eq.(1) is:

$$\frac{1}{[B]} = \frac{1}{[TAg_d]} + \frac{1}{K_{eq}[TAg_d]} \times \frac{1}{([TAb]-[B])} \qquad \text{Eq.(8)}$$

If I assume that the assay response A of a solid phase assay is directly proportional to [B] and if $[TAb] >> [TAg_d]$, such that $[TAb] \simeq ([TAb]-[B])$, this is equivalent to the approximation:

$$\frac{1}{A} = \frac{1}{k[TAg_d]} + \frac{1}{K_{eq}k[TAg_d]} \times \frac{1}{[TAb]} \qquad \text{Eq.(8a)}$$

where k is simply a proportionality constant converting antigen concentration to assay response units.

Plotting 1/A on the ordinate against 1/[TAb] on the abscissa will then give a line whose intercept with the ordinate (i.e at infinite antibody concentration; 1/[TAb] = 0) is equal to the maximum assay response possible in the system, and whose intercept is on abscissa, assuming functionally monovalent binding of antibody (R.7.4.6), is equal to $-K_{eq}$.

The critical condition for being able to use this method in practice is the assumption that [TAb] can be used to approximate ([TAb] - [B]). This will only be the case if $1/K_{eq} >> [TAg_d]$. Figure 35 presents the results of some model calculations which show the consequences if this condition does not hold. The line labelled "free antibody" indicates the ideal curve (line) which would be obtained using any concentration of antigen if it were possible to measure the amount of free antibody in the system. The family of curves to

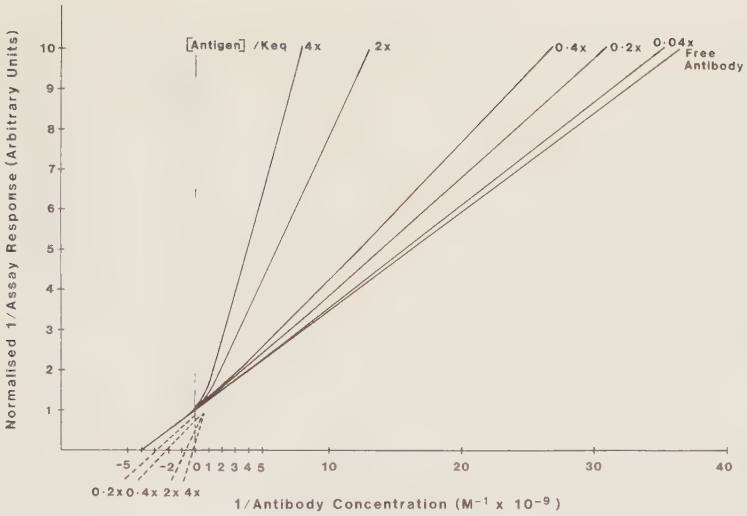

Fig. 35 **Double reciprocal plots of assay response against antibody concentration using values derived from model calculations, to show the effect of using the total, rather than the free, concentration of antibody to estimate the K_{eq} of a binding reaction.**

An arbitrary K_{eq} value of 4 10^{-9} M^{-1} has been assumed for the calculations. The maximum assay response has been set at 1.0. Using the law of mass action equation (Eq.(1), p.200), the amount of total antibody required to give the fractional assay responses of 0.1, 0.2, 0.3, 0.5, 0.74, 0.89 and 0.99 has been calculated for the antigen to $1/K_{eq}$ ratios indicated at the top of the figure. [Antigen]/($1/K_{eq}$) in the figure corresponds to $[TAg_d]/(1/K_{eq})$ in the text. Dotted lines to the left of the ordinate at 1/[TAb] = 0 show intercepts on the abscissa ($-K_{eq}$) obtained if a linear regressions were assumed and the curved part of the plots ignored.

the left of this line illustrates the errors which will result, at different $[TAg_d]/(1/K_{eq})$ ratios, if estimates of Keq are made using the reciprocal of the concentration of total antibody in the system rather than the reciprocal of the free antibody concentration.

It is clear that, as long as the antigen concentration remains less than half the $1/K_{eq}$ value, the curves do not deviate greatly from linearity and that extrapolation from the

values obtained at low total antibody concentrations (high 1/antibody values) will lead at most to a factor 2 error in estimates of K_{eq}. However, at antigen concentrations greater than $1/K_{eq}$, the approximation causes the plot to depart markedly from linearity at high antibody concentrations (leftmost two curves in fig. 35). At these high $[TAg_d]:1/K_{eq}$ ratios attempts to obtain estimates of K_{eq} by extrapolation (from the region of the curve where the relationship between the reciprocal of the assay response and the reciprocal of the antibody concentration approaches linearity) will causes large errors (> factor 10).

Armed with this knowledge, it is possible to investigate whether the desired conditions are fulfilled by antibody binding in solid phase assay. Figs 39 and 40 in the following two sections show examples in which this approximate approach is obviously justified. However, if there is any doubt about the appropriateness of the method, it can be tested by altering the antigen concentration. If reduction of the antigen concentration in the assay by a factor 5 to 10 causes an alteration of the K_{eq} estimate by a corresponding factor, measurement is clearly being carried out in an inappropriate range. In contrast, if the K_{eq} estimates are relatively insensitive to antigen concentration, some confidence can be placed in them. Because the K_{eq} values obtained in solid phase assay may differ from those in homogeneous phase (see R.7.2, p.197 ff.), they are referred to in what follows as **apparent** K_{eq} values, although they are clearly the appropriate values to describe the equilibrium in the microtitre plate system.

R.7.4.4.1 Non-specific binding

Problems arise in trying to use this approach in practice, because at high concentrations of antibody non-specific binding effects occur. Thus the range of antibody concentrations and affinities which can be usefully used is

limited. I have already considered one aspect of this problem in section R.7.3.1.2.3. Namely, in sufficiently high concentrations, many antibodies appear to be able to bind to BSA in large amounts. This binding is of low affinity, it is negligible at low concentrations of antibody (perhaps better illustrated in fig. 37b than in fig. 25), but is of high capacity. As a consequence, once antibody has become bound in large amounts, the relatively small volumes of the wash steps in microtitre plate wells are probably insufficient to remove enough of the non-specifically bound antibody at each wash to make it undetectable when the final step has been reached. Non-specific binding of this sort is probably not only a property of solid phase assays in microtitre wells. I suspect that the forces involved in such binding are probably not immunological: candidate mechanisms are ion-exchange effects and other adsorption phenomena. If this interpretation is correct, even a Western blot would be expected to stain completely by exposure to a sufficiently high concentration of antibody.

The kind of binding reported in section R.7.3.1.2.3 does not appear to be restricted to BSA. The monoclonal antibodies which have been tested up to now on plates coated with haemoglobin have given equally high, if not higher, levels of non-specific binding.

A still higher level of non-specific binding than that occurring to BSA is seen when antibodies which have been raised against non-Drosophila antigens are tested against protein either from Drosophila nuclear sonicates or soluble extracts of RNA associated protein from Drosophila nuclei. These antibodies can be shown by other methods (indirect immunofluoresence on Kc cells or Western blotting) not to cross-react with Drosophila antigens. The level of binding can be as much as 3 times greater than that to BSA. This can be seen by comparing fig. 36a and fig 36b which show titrations of the ELISA response to a Drosophila nuclear

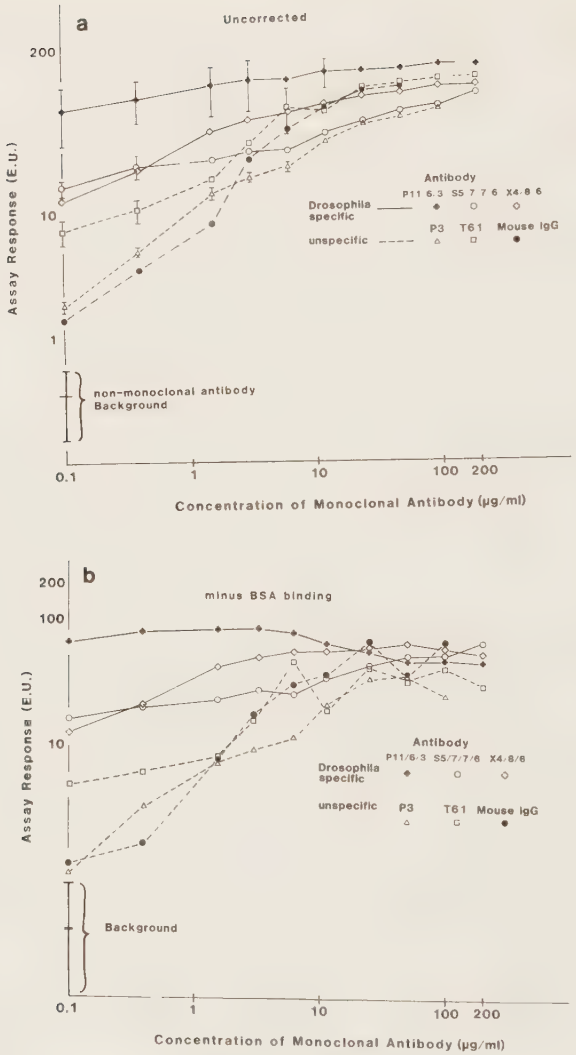

Fig 36 **Non-specific binding of mouse IgG and non-specific and specific monoclonal antibodies to a Drosophila Kc cell nuclear protein extract at high (> 1 µg/ml antibody) concentrations using ELISA**

(for figure legend see next page)

extract of three such non-specific antibodies (shown by dashed lines in figure: T61, directed against a chicken non-nuclear membrane antigen; P3 myeloma, antigen unknown; mouse IgG, various antigens) before and after "correction" for non-specific binding to BSA. The "correction" only removes 20% - 30% of the non-specific binding signal to the Kc material. For further comparison, the binding of 3 specific antibodies (P11/6/3, S5/7/7/6 and X4/8/6, solid lines) is also shown. At low antibody concentrations, in either the presence or absence of any "correction" the binding of the non-specific antibodies is lower that of the specific ones, satisfying the discriminatory criterion of specificity. At high concentrations, however, in the absence of "correction", the non-specific antibodies give an assay signal which is higher than that of two of the specific anibodies and is approaching the saturation value for the detector. Even "correcting" for nonspecific binding to BSA (fig. 36b, values shown in fig. 25

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(legend to fig. 36 continued from previous page)

Double logarithmic plot of ELISA response against antibody concentration.

Panel a: Observed ELISA response

Panel b: ELISA response "corrected" for non-specific binding of antibodies to BSA. At the corresponding concentration of antibody, the values presented in fig. 25 have been subtracted from the response using Drosophila protein shown in panel a.

Wells were coated with antigen by incubation with 1.5 µg of protein contained in an extract obtained by treating Drosophila embryo nuclei with RNAase and 0.4M NaCl and taking supernatant fractions after high speed centrifugation in sucrose gradients (< 10S fraction, kindly provided by M.FRASCH) for 4hr at room temperature. The coated wells were incubated with the concentrations of antibody indicated on the abscissa for 17 hr at room temperature and with phosphatase labelled detector for the same period of time. Antibodies not reacting specifically with Drosophila antigens are indicated by dotted lines and those binding specifically by solid lines. The identity of the antibody giving rise to each curve is indicated by the key on the figure. The error bars indicate the 95% confidence limits of the E.U. regression slope (panel a) or of the difference between two slopes (panel b), calculated from 4 or 5 time points. Only representative error estimates have been shown, to keep the figure comprehensible: to a first approximation, the observed variation in responses with similar mean E.U. values was the same.

have been subtracted from the corresponding values in fig. 36a) only reduces the assay response from the non-specific antibodies so that it now equals that of the specific ones rather than exceeding it. Since the Drosophila protein would be expected to occupy the greater proportion of the area of the well, so that little BSA would be expected to bind as a result of the BSA-block step, the appropriateness of the BSA binding "correction" is, in any case, doubtful. This does not, of course, rule out the possibility that the mechanism of non-specific binding, both to BSA and to Drosophila protein may be similar, especially when it is remembered that the amount of adsorbed Drosophila protein will be greater than the amount of BSA adsorbed (see previous section).

The concentration at 50% maximum assay response (approx. 10 µg/ml) of the non-specific antibodies shown in fig. 36a indicates a maximum affinity of these antibodies for their ligands of 10^7 M^{-1}. However, it is likely that the concentration of detector is responsible for the saturation observed in the the figure (data not shown), suggesting that this binding may be of still lower affinity.

The use of IRMA and sonicated nuclei gives similar results, as fig. 37 illustrates. Two experiments using different amounts of adsorbed antigen are shown (see figure legend). Although a different antigen mixture is being used for the IRMA and ELISA assays, the results of experiment 2 of the IRMA are very similar to those shown for ELISA in fig. 36a. In the IRMA assay shown in fig. 37a the non-specific binding by mouse IgG becomes greater than that by the specific S5 antibody at an antibody concentration of 2 µg/ml compared to a value of 3.5 µg/ml for the ELISA assay shown in fig. 36a. Fig. 37b shows that correction for binding to BSA does not greatly alter this picture and also that, at low concentrations of antibody, the binding of antibody to BSA is

almost negligible. More interestingly perhaps, the binding of the 32-5B6 antibody in experiment 2 shows that there are some non-specific antibodies which bind to Drosophila nuclear protein very much less well than mouse IgG and presumably therefore, by analogy with the results shown in fig 36, much less well than either of the monoclonal antibodies T61 or P3. This indicates that even in their non-specific binding properties at high concentrations there are differences between individual antibodies. This conclusion is supported by the results of FRASCH 1981, which show (his fig. 2) that there appears to be a continuum of binding affinities from extremely high affinity ($> 10^{11}$ M^{-1}) to even lower affinities than those I reported here.

These observations raise the question of whether the assay response by specific antibodies may not also contain a non-specific component. It is difficult to answer this question definitively. At first sight, it appears that the contribution of any non-specific component to the binding of either the S5 or X4 antibodies must be less than that by T61 antibody or mouse IgG, because the assay response at high antibody concentrations for the specific antibodies is less than for either of the non-specific antibodies. However, as I show in R.8.3 the efficiency with which individual antibodies are detected by detector may also vary by as much as a factor 4 to 10, and this might also contribute to the apparent difference. It is noteworthy in this respect, that the relative magnitude of the response to the the different antibodies at high concentrations roughly parallels the relative response that is obtained when the antibodies are adsorbed directly to the well surface.

Despite these problems, it does appear possible to obtain approximate estimates of apparent affinities of mononoclonal antibodies (see next section, R,7.4.4.2).

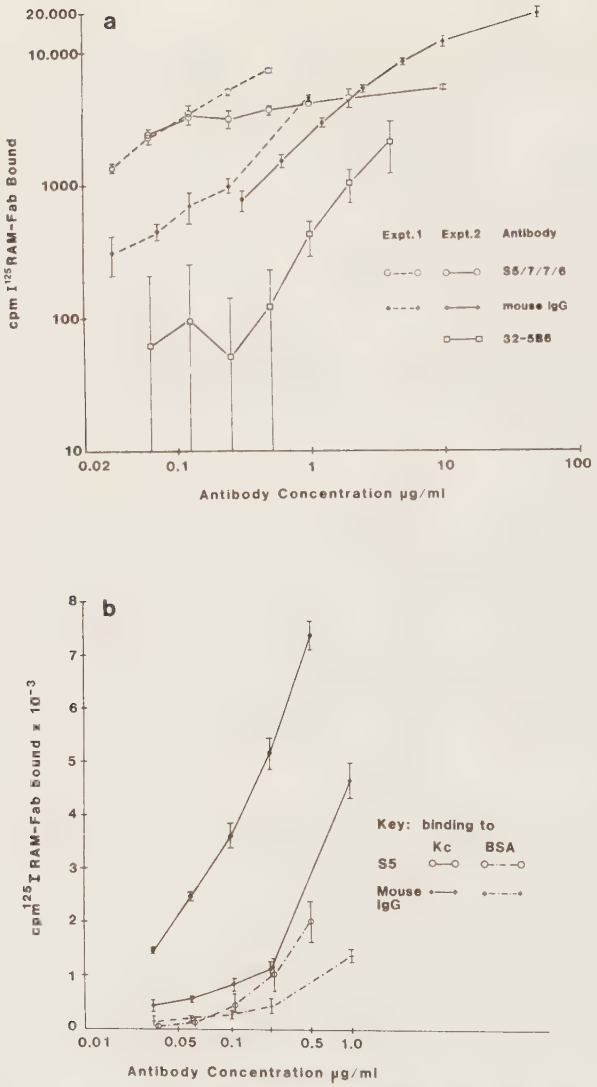

Fig. 37 **Non-specific binding of mouse IgG and monoclonal antibodies to Drosophila Kc cell nuclear protein at high (>1µg/ml) antibody concentrations assayed by IRMA**

(for legend see next page)

The conclusion to be drawn from the results presented in this section is clear: solid phase assay in microtitre plates will only yield trustworthy data about the presence and amount of specific antigens adsorbed from complex mixtures if the antibody concentration is kept below an empirically determined limit of about 1 µg/ml. This consideration is obviously important if the detection of false positives in screening hybridomas for antigen reactive clones. Although the standardisation assay for the amount of IgG discussed in the next chapter (R.8) is imprecise, adjusting unknown antibody concentrations to "1 µg mouse IgG equivalents/ml", will result in true antibody concentrations of between 0.5 and 5.0 µg/ml being used for screening and has proved effective (FRASCH 1981 and FRASCH pers. comm.) for screening. By using still lower concentrations of antibody, it would be possible to automatically limit selection to highly affine antibodies.

R.7.4.4.2 Antibody affinity and saturation binding of antigen

The other problem which I discussed at the beginning of this chapter was how to determine the number of antibody

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(legend to fig. 37, continued from previous page)

Panel a: double logarithmic plot of results of two separate experiments. In Expt. 1 100 µl sonicated Drosophila nuclei (10 µg protein) was incubated in microtitre plate wells for 1 hr. at 0°C. In Expt. 2 the same amount of sonicated nuclei was used, but was transferred to the wells from ice and incubated for 20 min at room temperature. After adsorption antigen was titrated with the antibody indicated in the Key using standard IRMA procedures. Input ^{125}I RAM-Fab was 1.26 $\pm$ 0.01 10^5 cpm or 1.25 $\pm$ 0.01 10^5 cpm for experiments 1 and 2 respectively. Data points are means of sextuplicate values (Expt. 1) or triplicate values (Expt. 2) and have been corrected for binding of ^{125}I RAM-Fab to Kc (105 $\pm$ 13 cpm for Expt. 1, 597 $\pm$ 70 for Expt. 2). $\pm$ indicates 95% confidence limits of mean, error bars indicate 95% confidence limits of difference between treatment and background.

Panel b: linear-log plot of assay response against logarithm of antibody concentration, showing same data as in panel a, experiment 1 and also binding of antibodies to BSA.

molecules bound per well when antigen is saturated with antibody. There are two aspects to this problem: how to determine the absolute number of molecules bound, which involves characterising and calibrating detector binding, which I discuss in the next section, and how to determine in indirect assay if antigen is saturated by first (usually monoclonal) antibody. The occurrence of non-specific binding effects could pose problems for the determination of the latter quantity. While the use of low concentrations of antibody affords maximum selectivity in the assay response, it might also compromise the accuracy with which affinity could be estimated if reliable data could not be gathered over a wide range of antibody concentrations because of superimposed non-specific effects. It might be difficult to decide whether non-linear double reciprocal plots were the result of unsuitable $[TAg_d]/(1/K_{eq})$ ratios or non-specific binding effects (or both). This would pose a dilemma because, if the non-linearity were caused by unsuitable amounts of antigen, saturation would be best estimated by making a smooth curve extrapolation of the double reciprocal plot to the intercept on the ordinate, and this would best be done by gathering much data at high assay response levels (cf. fig. 39b). In contrast, if the non-linearities result from non-specific effects, then (providing the $[TAg_d]/(1/Keq)$ ratio was suitable) saturation of specific binding would be best estimated by linear extrapolation of binding at low antibody concentration and low assay response levels, even though such a weighting of the regression estimate might cause undesirable innaccuracies.

Fortuitously (?), using the approximate method outlined at the beginning of this chapter (R.7.4.4), all the specific antibodies which have been examined up to now, with the exception of the X4 antibody assayed in the warm (see R.7.4.7), have given good linear fits to when assayed over the concentration range 0.01 µg/ml to 1 µg/ml (see fig. 40).

Table 31 summarises a number of estimates of affinity which have resulted.

Antibody	Apparent Affinity ($M^{-1} \times 10^{-9}$)	95% confidence limits	Data source
P11/6/3	2.5	1.7 - 3.3	36 extract
	4.1	3.7 - 4.6	40 extract
	5.4	1.9 - 10.1	extract/5
	10.0		FRASCH 1981 nuclei
P46/3	100		FRASCH 1981 nuclei
S5/7/7/6	0.9	0.4 - 1.4	37 nuclei
	1.9	1.2 - 2.6	36 extract
	2.9	1.7 - 4.3	extract
	9.3	5.9 - 14.0	extract/5
T7/3/3/4	1.2	0.8 - 2.0	extract
X4/8/6	0.4	0.03 - 0.8	36 extract

Table 31 **Apparent affinity of monoclonal antibodies**
The estimates were made by titrating assay response against antibody concentration and calculating the linear regression of 1/assay response vs 1/[antibody]. No attempt was made to weight the data to compensate for the overproportional influence of low assay response, low antibody concentration values on the regression estimate. A number before the data source indicates that the data is displayed in the figure with that number. Extract refers to an RNAase treated nuclear extract (see legend to fig. 36), extract/5 indicates that 1/5 the standard amount of this extract was used to coat the well with antigen.

There is a considerable spread of estimates where multiple determinations have been made (factor 4 for P11, factor 10 for S5) but, considering the wide range of conditions under which these estimates were gathered and the problems of non-specific binding, the agreement is suprisingly good. All the antibodies tested (except X4) have affinities greater than 10^9 M^{-1}. This level of affinity was one of the factors contributing to the ability to be able to carry out such measurements in the first place. It meant that data could be gathered at high assay response levels (>50% of the -

specific - maximum) over about a 10 fold region of antibody concentration (below 1.0 µg/ml), without great danger of interference from non-specific effects. In log-linear plots of titration curves such as those shown in figs 36 and 37, this property is manifested by a prominent plateau in the assay response values extending to low (below 0.1 µg/ml) antibody concentrations (not shown, but see figs. 1 and 2 in FRASCH 1981). The other factor making the estimates possible was that the linearity of the double reciprocal plots for assay response values in the range above 50% of the (specific) maximum for all the antibodies indicated that the "concentration" of adsorbed antigen must be considerably less than the K_{eq} value. The cause of the variability in the S5 K_{eq} estimates documented in table 31 is unclear. The increase in the K_{eq} with decreasing antigen concentration (extract vs extract/5) is the opposite of the effect that would be expected if the $[TAg_d]/(1/K_{eq})$ ratio were too high. (cf fig. 35).

Information about affinity, obtained using this approximate method, is useful in two ways. Firstly, it directly sets an upper limit to the amount of antigen from the mixtures that can have been adsorbed in immunologically detectable form. Secondly, at least using the antibodies which have thus far been extensively titrated are representative, it means that using the standardised "1 µg/ml mouse IgG equivalent" concentration of antibody, the adsorbed antigen from the complex mixtures tested was certainly saturated, allowing the quantitative estimates of antigen amounts outlined in the next section (R.7.4.5) and in chapter R.9.2 to be made.

R.7.4.5 Characteristics of detector (anti-antibody) binding

At the beginning of this chapter, I suggested that the range of measurement in IRMA was limited because the maximum amount of radioactively labelled detector would be insufficient to saturate the antibody to which it bound. For pure antigen, there can be no argument about the correctness of this suggestion. The number of antigen molecules which can bind to a well surface is at least 100 fold greater (see table 28) than the maximum number of radioactively labelled detector molecules which could possibly (indirectly) bind to them. If more than one antibody is able to bind per antigen molecule and if, as practice showed, only 20% of the radiolabelled detector is able to bind to any one kind of antibody (see R.7.3.2) under solid phase assay conditions, the insufficiency of the amount of radiolabelled detector with respect to bound antibody will be still greater.

When complex mixtures of antigens are assayed, whose individual components probably make up only a fraction of 1% of the total number of molecules in the sample (as the ability to use the approximate method for measuring antibody affinities, with its low $[TAg_d]/(1/K_{eq})$ ratio requirement implies), it could be argued that, if the concentration of immune complexes is sufficiently small and the affinity of radiolabelled detector sufficiently great, detector might be able to saturate all complexes. Empirical testing of the effect of altering the concentration of radiolabelled second antibody in IRMA showed that this was not the case.

Fig. 38a shows that for the antibodies P11, P12, P46, S5 and T7, as the assay response is titrated with respect to the amount of detector, it continues to increase within the range tested. However, for all except the P11 antibody, the rate of increase with respect to detector concentration is clearly falling off at high detector concentrations indicating that saturation is being approached. The data shown in fig. 38 has not been corrected for background (binding of radiolabelled

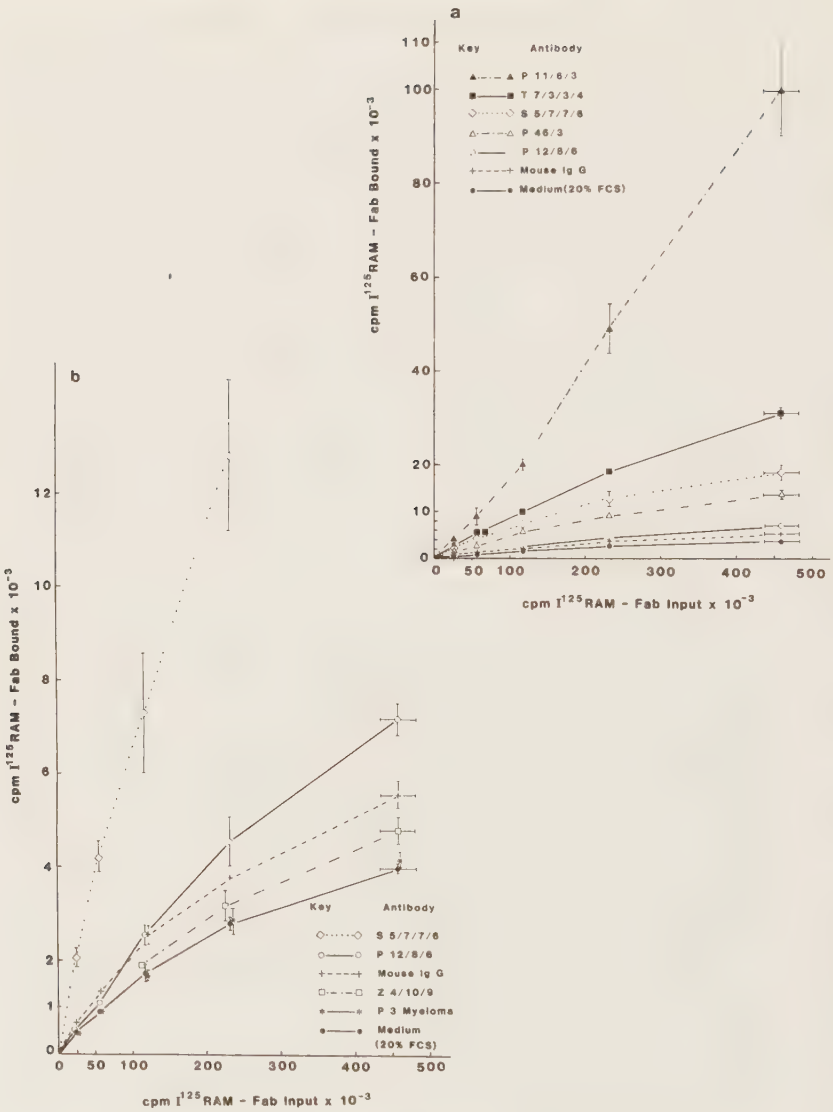

Fig. 38 **Dependence of IRMA response on the input of radiolabelled detector**

(for legend see next page)

detector to Drosophila Kc nuclear components in the absence of specific antibody), because I wish to show that, even at high levels of radioactivity, non-specific radioactive binding is not a serious source of error. Indeed, precision is increased by using higher concentrations of detector. Both the specific response and the non-specific background increase as function of increased radioactive input, but the absolute value of the difference between background and specific binding increases at a greater rate than the variance of the difference. Thus, at high levels of labelled detector, specific signal and non-specific background can be more reliably distinguished from one another. This is perhaps most clearly seen in fig. 38b in which part of the data in the lower part of fig. 38a is shown with the scale of the ordinate expanded. Note that, under the conditions used for this assay, the P3 myeloma antibody does not give rise to assay signal which significantly exceeds background, whereas some component(s) of mouse IgG clearly do bind at a level not greatly but significantly higher than background.

As I indicated in the previous section, at a concentration of 1.0 µg/ml, all the antibodies for which data are shown in fig. 38 will be saturating their respective antigens. The use of double reciprocal plots can then also be used to estimate the amount of detector bound at saturation.

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(legend to fig. 38 continued from previous page)

A standard IRMA assay using 10 µg sonicated Drosophila Kc cell nuclei as a source of antigen and 1 µg/ml monoclonal antibody, but using the amounts of detector (^{125}I RAM Fab) indicated on the abscissa. Specific activity of detector was 33 mCi/mg.

Panel a: Response of all antibodies tested shown on the same scale.

Panel b: Detail of panel a to show precision of measurement at low levels of bound detector.

Data points are means of quadruduplicate values. Input count estimates are the means of 9 replicate samples except for the 4 10^5 cpm estimate which was made from triplicate samples. The vertical and horizontal error bars indicate the 95% confidence limits of the respective means. Error bars have been omitted where they would make the data difficult to see.

As in the previous section total, rather than free, detector can be used as an approximation for the concentration of free antibody. Fig. 39a shows examples of such plots using the data for the S5 and T7 antibodies shown in fig 38. The linear fit is very good, indicating that the binding behaviour for these antibodies can be adequately described by standard binding theory. The amount of radiolabelled detector required to saturate binding of the P11 antigen could not be estimated on the basis of the data in fig. 38 because the the binding of detector to P11 showed a sigmoidal dependence on total detector concentration and only tended to saturate at very much higher concentrations than those used. However, by altering the specific activity of the radiolabelled detector by dilution with unlabelled RAM-Fab and extending the titration with detector to an amount equivalent to 4 10^6 cpm/well it was possible to estimate saturation by smooth curve extrapolation of a double reciprocal plot. Appropriate correction and recalculation of the data on the basis of free rather than total detector (cf. Eq. (8) and Eq.(8a) at beginning of R.7.4.4) showed that the curved form of the double reciprocal plot did not result from using total detector cpm as an approximation for unbound detector (data not shown). It is more likely that the curvature is caused by two subsets of the detector molecules binding to the P11 antibody with different affinities.

The stoichiometry of ^{125}I RAM-Fab binding to the monoclonal antibody P11/6/3 in normal IRMA conditions was estimated by using ^{125}I labelled P11/6/3 and directly measuring the amount of ^{125}I RAM-Fab bound to it. The ^{125}I labelled P11/6/3 preparation contained carrier protein (BSA), and was of such high specific activity that it was not possible to directly measure the recovery of protein after labelling. A nominal specific activity of 18 mCi/mg can be calculated from the recovery of radioactivity from the column used for the preparation of the labelled antibody, if 100% recovery of

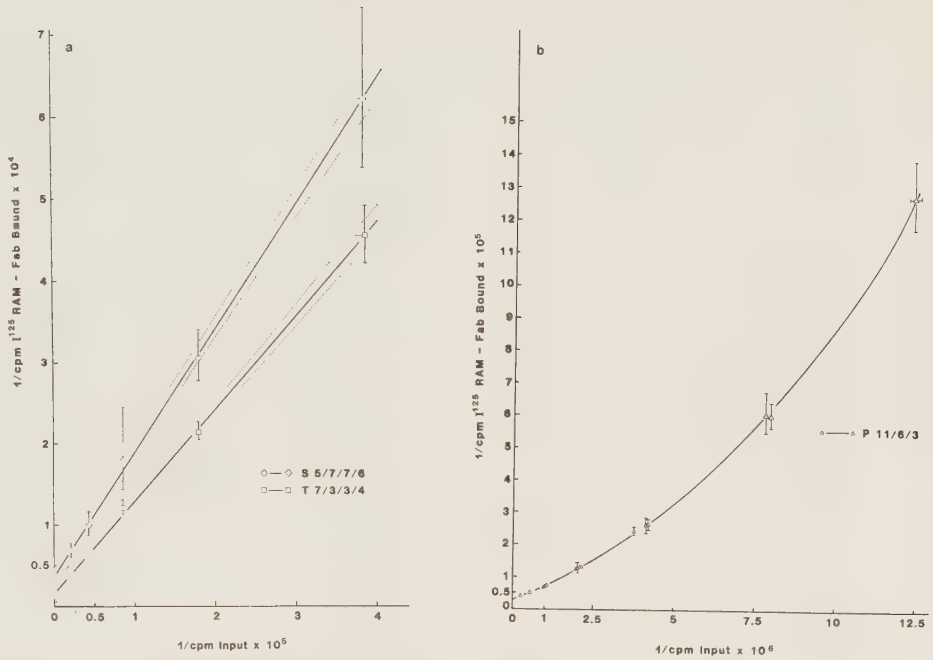

Fig. 39 **Double reciprocal plots of bound detector against detector input used for estimation of maximal detector binding**

Panel a: S5 and T7 data from fig. 38, least squares linear regression fit. Dashed lines, 95% confidence limits of estimates of 1/bound cpm based on the regression.

Panel b: plot of P11 data from a separate experiment fitted by inspection with a smooth curve.

protein is assumed. As a control for this estimate, an amount of unlabelled P11 antibody equivalent to that estimated on the basis of its nominal specific activity was tested in parallel. The results of these experiments are shown in table 32 (p.337).

It is clear from the difference between the amount of binding of ^{125}I RAM-Fab to nominally equivalent amounts of labelled and unlabelled P11 (compare columns 3 and 4 in table 32) that the concentration of active ^{125}I labelled antibody must be less than that calculated on the basis of its nominal specific activity. A comparison of the data from normal and P11 depleted nuclei shows that the ratio of ^{125}I RAM-Fab bound to labelled and unlabelled P11 is the same in both cases (1.5, column 5 of table 32) indicating that a systematic error has been made in the estimate of active P11. There are 3 logical possible explanations for this discrepancy.

1) The nominal specific activity value could be in error (e.g because the recovery of protein from the column used for the preparation of radiolabelled P11 was less than 100%)

2) A proportion of the P11 antibody might have become inactivated by the labelling procedure so that it was unable to bind antigen

3) All the P11 antibody might be active and have the correct nominal specific activity, but as a consequence of iodination 30% of the iodinated P11 might no longer be recognised by RAM-Fab.

On the basis of the data presented in table 32 alone there is no way to decide which of these possibilities is correct. Depending on which possibility is chosen different values for the stoichiometry of ^{125}I RAM Fab binding will result. The possible solutions which may be calculated are listed in table 33. It is clear from this table that, while there are differences, the stoichiometry estimate is not very

	TREATMENT NR.				
	1	2		3	
	^{125}I P11/6/3	^{125}I P11/6/3 + ^{125}I RAM-Fab	Difference between treatments 2 and 1 = 125 RAM-Fab bound to ^{125}I P11/6/3	Control unlabelled P11/6/3 + ^{125}I RAM-Fab	Ratio detector binding to unlabelled vs labelled P11
	cpm bound				
Normal nuclei	29,053 ± 644	57,907 ± 1864	28,854 ± 1864	43,892 ± 292	1.5 (1.4 - 1.6)
P11 depleted nuclei	1413 ± 58	4102 ± 220	2689 ± 210	4086 ± 293	1.5 (1.3 - 1.8)

Table 32 **Parallel direct and indirect measurement of monoclonal antibody binding to P11 antigen**

P11 antigen was adsorbed to the surface of PVC microtitre plate wells from sonicates of nuclei prepared either from normal Kc cells or from cells which had been starved in the course of labelling with ^{35}S methionine (= P11 depleted nuclei). 10 µg or 7 µg protein (Bradford) respectively was incubated per well. The wells of treatments 1 and 2 (see table heading) were incubated with 3.22 ± 0.07 10^5 cpm ^{125}I P11/6/3 with a nominal specific activity of 18 mCi/mg (=11 ng), the wells of treatment 3 with 11 ng unlabelled P11/6/3. P11 antibody in all treatments was made up in Iscoves medium containing 10% FCS at 5 10^{-10} M. Wells were incubated with medium alone (for non-specific binding correction). After binding for 12 hours, the wells were washed (normal IRMA) and exposed overnight to either 2.34 ± 0.02 10^5 cpm ^{125}I RAM-Fab with a specific activity of 33 mCi/mg (=6.6 ng), treatments 2 and 3 or to 6.6 ng unlabelled RAM-Fab treatment 1 at a concentration of 4.4 10^{-10} M. All values are means of 12 replicate values and have been corrected, as required, for P11/6/3 non-specific binding (to BSA) of 120 ± 30 cpm and ^{125}I RAM-Fab non-specific binding to Kc cells (253 ± 40 cpm). ± indicates 95% confidence limits.

sensitive to the assumptions used for its calculation. It may be safely assumed that, under usual IRMA conditions, at very low levels of bound P11 antibody (< 50 pg), the ratio of second antibody to first antibody is between 1 and 2, whereas when the amount of bound P11 is about 1 ng the ratio is less than unity. The estimate of maximum detector binding obtained from fig. 39 (table 34), indicates that, at saturation, 4 (4.18 measured) detector molecules will bind to the P11 antibody.

These findings are direct confirmation of the limiting nature of detector binding. They also show that the use of indirect technique in IRMA does not result in any molecular

	P11 depleted nuclei			normal nuclei		
Assumption	Absolute amount antibody bound (ng)		molar ratio detector: monoclonal	Absolute amount antibody bound (ng)		molar ratio detector: monoclonal
	P11/6/3	RAM-Fab		P11/6/3	RAM-Fab	
P11 iodination damage	0.050	0.052	1.0	1.01	0.48	0.48
P11 sp. activity in error	0.035	0.052	1.48	0.67	0.48	0.72
P11 antigenicity altered	0.05	0.078	1.55	1.01	0.67	0.67

Table 33 **Binding stoichiometry of RAM-Fab to P11/6/3**
Calculated on basis of data in table 32 making different corrections for the low apparent specific activity of ^{125}I P11/6/3

amplification, except at extremely low levels of bound first antibody. However, it is at low levels of immune complexes, that increased sensitivity will be most useful.

It is difficult to decide how applicable the results for P11 are to the other antigens. The curved double reciprocal

plot shown in fig. 39b suggests that different determinants are recognised by detector molecules with different affinities and that possibly they are present in the detector population at different frequencies. The maximum stoichiometry of 4 is in agreement with this estimate. Since the detector was prepared against Fab fragments, the determinants are likely to be distributed symmetrically on each of the two arms of the IgG molecule (i.e. 2 sites per arm). In contrast, the very good linear fit to the double reciprocal plot for the two IgG1 molecules examined, is most simply interpreted by assuming that detector binds to these antibodies with uniform affinity. Uniform affinity might mean that only a single antigenic determinant on each Fab arm of these antibodies is recognised by detector, it can, however, hardly be considered as conclusive evidence for such stoichiometry. Nonetheless, if this explanation of the difference between detector binding to the P11 antibody and to the other antibodies is correct, it could in part account for the different sensitivity of different antibodies in the standardisation assay (see R.8.3). The amount of detector which could bind to different monoclonals would vary, depending on whether it possessed 2,4..6 antigenic determinants. This might explain why the sensitivities in this assay tend to cluster around particular values (table 38, R.8.3).

Given these stoichiometries and estimates of the maximally bound amount of detector, it is possible to calculate the amount of monoclonal antibody bound to adsorbed antigen and hence, assuming a 1:1 monoclonal:antigen stoichiometry, the amount of adsorbed antigen. Derivation of the estimates of bound antibody for the five monoclonal antibodies P11, P12, P46, S5 and T7 is shown in table 34. The first two lines of this table present estimates of the maximum number of detector counts bound, estimated by the use of double reciprocal plots. Notice the good agreement between the S5 and T7 estimates from two separate experiments. The means

	ANTIGEN				
	S5	T7	P46	P11	P12
Expt. 1 max. cpm bound x 10^{-3}	24.7 (16.9-45.9)	58.5 (32.6-292)	14.7 (8.0-80)	-	3.5 (1.5-10)
Expt. 2 max. cpm bound x 10^{-3}	29.4 (23.3-39.5)	50.9 (30.3-157)	-	333	-
Mean Estimate max cpm bound x 10^{-3}	27.0 (16.9-45.9)	54.7 (30.3-292)	14.7 (8.0-80)	333	3.5 (1.5-10)
Max amount detector bound moles x 10^{15}	3.4 (2.1-5.8)	6.9 (3.8-37)	1.8 (1.0-9.7)	42	0.4 (0.2-1.2)
Best estimate moles monoclonal bound x 10^{15} (stoichiometry correction)	1.7 (1.0-2.9)	3.5 (1.9-18.5)	0.9 (0.5-4.8)	11	0.2 (0.1-0.6)
Molecules monoclonal bound = antigen adsorbed x 10^{-9}	1.0 (0.6-1.8)	2.0 (1.1-11)	0.5 (0.3-3.0)	6.7	0.1 (0.1-0.4)
K_{eq} detector M^{-1} x 10^{-9}	1.8 (0.9-3.4)	1.0 (0.3-2.5)	2.5 (0-6.3)	-	-

Table 34 **Estimates of the amounts of immunologically detectable antigen adsorbed to PVC microtitre plate under normal solid phase IRMA conditions**

See text for explanation. Figures in brackets are 95% confidence limits except for P46 and P12 estimates, which are 90% confidence limits.

of these estimates have been divided by the specific activity of the detector (5.28 10^{18} cpm/mole at 1.73 mole ^{125}I/ mole IgG), to give the moles of detector bound (line 4). Correction for the stoichiometry of the detector:monoclonal binding reaction (2 for all IgG1 monoclonals, 4 for the IgG2a molecule P11) then gives the moles of bound monoclonal antibody, or

adsorbed antigen (line 5). Multiplication by Avagadro's number converts moles to the number of molecules of adsorbed antigen (line 6) which are detected. The final line (line 7) of table 34 lists estimates of the apparent K_{eq} of detector for monoclonal antibody in this system.

There is reasonable agreement between these figures and the theoretical estimates of assay sensitivity made in section R.7.2.1.3. The P12 antigen, which gave the lowest detectable response in this series of tests, is estimated to have been generated by 10^8 molecules of adsorbed P12 antigen. Assuming a detector affinity (K_{eq}) of approximately 2 10^9 M^{-1}, this may be compared with the figure of 1.2 10^8 given in table 20 for the minimum amount of detectable antigen assuming a 1:1:1 antigen:antibody:detector complex and a detector affinity of 10^9 M^{-1}. Using 100,000 cpm of detector, only about 1000 cpm or 1% of the counts are bound to adsorbed antigen (fig. 38b) indicating an antigen:antibody:detector ratio of 1:1:0.7.

If the interpretation of the difference between the shape of the detector binding curves to the IgG1 antibodies (S5,T7) and to IgG2a (P11) is correct, it follows that there is likely to be a proportion of the detector population which is able to bind IgG2a but not IgG1 molecules. This means that the concentration of molecules actively able to bind IgG1 will be less than the total concentration of detector. To obtain a correct estimate of apparent K_{eq} for detector would then require plotting the reciprocal of this reduced concentration in double reciprocal plots. Failure to make such an estimate will result in an underestimate of the true apparent K_{eq}, and this is probably the case for the estimates in table 34. The 3 (under) estimates of the affinity of detector for IgG1 which were obtained differ by a factor 2.5, but the confidence limits of all three overlap. It is therefore possible that detector binds to all 3 antibodies, and perhaps to all IgG1 molecules, with identical affinity.

Finally, the estimates of antigen amounts listed in table 34 show that the use of the $[TAg_d] < (1/K_{eq})$ approximation for the determination of apparent binding constants (Eq.(8a) p. 318) is justified. For example, the amount of T7 antigen adsorbed (line 6 of table 34) is $2\ 10^{9}$ molecules, corresponding to a $3\ 10^{-11}$ M "concentration" of adsorbed antigen with respect to the 100 µl volume of solution with which the well is usually incubated. The $1/(K_{eq})$ for T7 binding is $8.3\ 10^{-10}$ (table 31) giving an $[TAg_d]/(1/K_{eq})$ ratio of 0.03. Reference to fig. 35 will show that this ratio has only a very small effect on the accuracy with which apparent K_{eq} can be determined.

R.7.4.6 Monovalent binding of P11 antibody in solid phase assay

Fig. 40 shows the double reciprocal plots of the ELISA assay response of intact P11 antibody and of its Fab fragment against antibody concentration. While the linear fit to the binding data of the intact P11 molecule is clearly acceptable, the large deviations of some of the experimental data from the most probable linear fit make it questionable whether a linear relationship is the appropriate way to describe the Fab data. Nonetheless, I would argue that this model is correct, because the binding data to 1/5 the amount of antigen for both intact P11 and P11-Fab gave regressions in double reciprocal plots very similar to those shown in fig. 40 (data not shown). Furthermore, although the other set of Fab-binding data also showed a lot of scatter, the pattern differed from that seen in fig. 40. This indicates that the large deviations from the fitted regression, while highly undesirable, because of the imprecision they generate, do not provide reliable evidence for a curvilinear relationship between the reciprocals of assay response and antibody concentration, which would indicate binding behaviour not simply governed by the law of mass action.

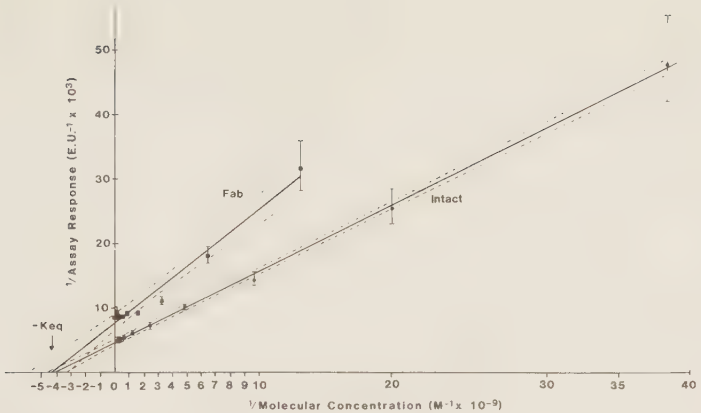

Fig. 40 **Double Reciprocal Plot of ELISA Assay response against antibody concentration for intact P11 antibody and its Fab fragment.**

Details of assay as for fig. 36. Solid lines show best fit regressions calculated by the method of least squares. Dotted lines show 95% confidence limits of 1/(E.U) values estimated from the regression. Error bars are 95% confidence limits of E.U. calculated from 4-6 time points.

Although the intercepts on the ordinate for each of the two regressions are different, indicating that the maximum assay response is different for intact antibody and its Fab fragment, the intercepts on the abscissa (=-K_{eq} apparent) are, within experimental error, the same. The simplest explanation for this result is that the intact P11 molecule binds monovalently to its antigen, and that therefore both Fab and intact antibody bind to antigen with the same affinity (same K_{eq}). Note that, assuming this explanation, although the concentration of Fab arms in an intact antibody preparation is twice that of a Fab preparation at equivalent molecular molarities, it is the concentration of intact IgG **molecules** and not of their Fab arms which is used to derive the K_{eq} which must be compared with that of the Fab fragments. This is because 2 moles of Fab arms are removed from solution every

time an intact IgG molecule binds, even though only one Fab arm is actually responsible for binding. i.e. formally, from the law of mass action, denoting free Fab fragments by Fab, Fab arms attached to intact IgG by Fab', intact antibody by Ab and antigen by Ag, for intact antibody molecules:

$$K_{eq} = \frac{[Ab.Ag]}{[Ab_{free}][Ag_{free}]}$$

and for Fab

$$K_{eq} = \frac{[Fab.Ag]}{[Fab_{free}][Ag_{free}]}$$

At 50% binding of antigen the concentration of immune complexes [Ab.Ag] or [Fab.Ag] = $[Ag_{free}]$, and if K_{eq} is equal for Fab and the intact molecule, then the following equality results:

$$K_{eq} = 1/[Ab_{free}] = 1/[Fab_{free}]$$

but [Ab]= 2 [Fab'] and [Ab.Ag] = [Ag.2Fab'], and thus

$$K_{eq} = 1/[Ab_{free}] = 1/2\ [Fab'_{free}] = 1/[Fab_{free}]$$

i.e. plots of 1/Assay response against 1/[Fab'] would (and do) give a K_{eq} estimate for the Fab arms of an intact molecule which is half that of the whole molecule.

If the P11 antibody were to bind by a bivalent mechanism, the apparent K_{eq} of the binding reaction for the intact molecule would be expected to differ from of the Fab fragment by a factor 10 - 100 or more (see R.7.2.2.2). This is clearly not the case, and such a possibility falls far outside the

region of experimental imprecision. If only a part of the P11 antibody population were to bind bivalently, then a biphasic (or curved) double reciprocal plot would be expected. However there is no evidence for such an effect in the data shown in fig. 40.

Although a salt and nuclease treated extract was used as the source of antigen for the experiment shown in fig. 40, the observed apparent affinity of P11 in this experiment was the same (to within a factor 2, see table 31, p.329) as that observed using sonicated whole nuclei as a source of antigen.

A reasonable conclusion from the above considerations is that, whether antigen is derived from whole nuclei (in which P11 is present mainly in particulate form, RISAU et al., 1983)) or from salt extracts of nuclei (in which the P11 antigen is probably present only in monomeric form, RISAU et al., 1983), almost all P11 antibody binds to its antigen, in solid phase assay via only a single arm. The apparent affinity of the intact P11 molecule for antigen is thus governed directly by the site binding constant.

A further feature of the data shown in fig. 40 requires comment. Although the K_{eq} of P11-Fab and intact P11 is the same, the assay response at saturating levels of antibody is twice as high for intact antibody as for Fab. At low levels of antigen (1/5 that used to obtain the results shown in fig. 40), the ratio is still greater: the signal from intact antibody is approximately 6 fold higher than that from Fab. There is a simple explanation for this observation. Namely, that at most 1/6 of the total number of detector binding sites on the intact IgG molecule are present on a single Fab arm. At the high concentration of **antigen** used for the experiment shown in fig. 40, the concentration of detector is limiting (data not shown) for at least some of the determinants on the intact molecule and for this reason the full magnitude of the difference is not seen. The simplest distribution of

determinants on the intact molecule which would explain the above observations is 4 sites on the Fc portion of the molecule and 1 site apiece (identical) on each of the Fab arms. Logically, of course, any multiple of this distribution (e.g. 8:2:2) would also be possible, but since other investigators (cf. ARNDT-JOVIN et al., 1983) assume approximately 5 binding sites as an average number of different sites recognised by an anti-antibody, the simplest distibution is also the most plausible.

The specificity of the detector used for IRMA (anti-Fab) and in ELISA (anti intact IgG) is different, so there is no contradiction between the 4 site stoichiometry for P11 observed in IRMA and the 6 site stoichiometry observed in ELISA. Despite probably only having one binding site for detector, the Fab assay response can still be clearly seen. This indicates that molecular amplification is not neccessary to observe the ELISA signal. If some other monoclonal antibodies have less than 6 recognition sites for detector, this might also help to explain why, when antibody is bound directly to plastic, the signal from all other antibodies tested so far is lower than that from P11 (see R.8.3).

R.7.4.7 Post antigen adsorption effects of salt and temperature on assay response: differences between monoclonal antibodies

The response of individual antibodies to both salt and temperature varied. The effects were not limited to the adsorption phase of the assay (cf. R.7.4.2). Table 35 documents results, from a representative experiment, which show that salt, applied at different stages of the assay, affects the assay response to the antigens P11, X4 and T7. 2M NaCl clearly depresses the response of all 3 antibodies, although the extent of depression defined as:

$$\text{Assay depression} = \frac{\text{response without salt minus response with salt}}{\text{response without salt}}$$

varies by a factor of 3 from only about 25% for the P11 antibody to 75% for the X4 antibody.

Antibody	Control (Borate saline) (all incubations)	2M NaCl present from following step onwards: Detector binding	Monoclonal antibody binding	Antigen adsorption
		ASSAY RESPONSE IN E.U		
P11	203 ± 40 (4)	149 ± 19 (5)	155 ± 29 (5)	145 ± 13 (6)
X4	14.7 ± 1.2 (7)	4.0 ± 0.1 (8)	4.2 ± 0.1 (7)	3.7 ± 0.2 (8)
T7	8.8 ± 0.6 (7)	5.4 ± 0.2 (8)	5.5 ± 0.2 (7)	4.8 ± 0.2 (8)

Table 35 **2M NaCl depresses the assay response to three monoclonal antibodies**

ELISA assay response values under control conditions or with 2M NaCl included in the buffer during and after the indicated steps are shown. Details of the assay were as in the legend to fig. 36, except that after adsorbtion of antigen all subsequent steps were carried out on ice, and the incubation with phosphatase was for only 6 hours. The plate was returned to room temperature before the final wash which was made with buffer equilibrated at room temperature. The phosphatase reaction proceeded at room temperature in the usual way. ± values are 95% confidence limits. Figures in brackets indicate the number of time points from which E.U. were estimated.

The table also shows that such differences are greatly in excess of the measurement error associated with the assay. The second column of figures from the left of the table shows that the added salt can exert its effect even if it is only included during incubation with the detector. This is in contrast to the results reported in section R.7.4.2, in which the adsorption of antigens H1 or of S5-ag or T7-ag to solid phase was preferentially affected (the other steps of the

assay reported in R.7.4.2 routinely contained salt). There is no contradiction between the two sets of the results. The antigens whose assay is reported in R.7.4.2 were probably localised, at least in part, in high M.W. particles with low diffusion constants, whereas the antigens in the mixture used to obtain the results shown in table 35 were obtained from nuclei exposed to salt and probably existed mainly as individual molecules. At the very most they could have existed as subunits in small oligomeric proteins (M.W. $< 10^5$, see table legend for details antigen mixture preparation).

The discrepancy between the absolute E.U. value of the X4 assay response seen in this series of experiments and the severalfold higher values shown in fig. 36b is explained by the longer incubation period with phosphatase coupled anti-antibody in the latter experiments.

Not only salt has an effect on the relative response produced by the antibodies, but also temperature. Table 36 shows that although carrying out the assay at room temperature rather than in the cold had no detectable effect on either the P11 or the T7 antibodies, it reduced the assay response of the X4 antibody by a factor 3.

The combined effect of temperature and salt was additive, (or more correctly multiplicative). This is summarised in table 37 which shows the effect of salt and temperature expressed as the proportion of the maximal response obtainable from each of the three antibodies.

Logically, both effects may result either from a reduction in the binding of detector, from a reduction in the binding of monoclonal antibody to antigen or from a combination of both factors. It would require further experimentation to reliably establish which binding reaction was sensitive. However, the stronger response of the X4 antibody, relative to T7, in the standardisation assay in

Antibody	Assay conditions: COLD	ROOM TEMPERATURE	WARM/COLD
	Assay Response (E.U)		
P11	203 ± 40 (4)	202 ± 22 (4)	1.0 (0.74-1.37)
X4	14.7 ± 1.2 (7)	5.1 ± 0.1 (8)	0.35 (0.32-0.38)
T7	8.8 ± 0.6 (7)	8.0 ± 1.0 (8)	0.91 (0.74-1.1)

Table 36 **The ELISA response with the X4 antibody is lower at room temperature than in the cold**

Adsorption of antigen was at room temperature for both treatments. Thereafter assay details of cold treatment were exactly as described in the legend to table 35. For the room temperature treatment all steps were at room temperature, and the incubation time with detector was reduced to 3 hours so that E.U. values similar to those generated by cold treatment could be obtained. ± indicates 95% confidence limits, the single number in brackets the number of time points from which E.U. was estimated. The figures in brackets under the warm/cold ratios are worst case estimates of the ratio.

Antibody	TREATMENT: Salt only	Room temp. only.	Room temperature plus salt
	Assay response relative to control		
P11	0.73 ± 0.06	1.13 ± 0.13	0.86 ± 0.07
X4	0.27 ± 0.04	0.35 ± 0	0.08 ± 0.07
T7	0.60 ± 0.11	0.91 -*	0.54 ± 0.09

* single value

Table 37 **Relative assay response of 3 monoclonal antibodies as a function of salt and temperature**

Response is expressed as a fraction of the response obtained in the cold using borate saline for all incubations. Results calculated from pooled data of two separate experiments. From left to right the fractions shown are the means of 3, 2 and 6 determinations respectively. ± figures indicate 95% confidence limits or range if only duplicate values.

which the monoclonal antibody is adsorbed directly onto plastic, and which is carried out at room temperature, rather suggests that the monoclonal-antigen binding reaction is being affected. There is a precedent for a monoclonal antibody whose binding to antigen is worse in the warm than in the cold (MASON and WILLIAMS 1980).

These results are significant, because they show that even attempts to obtain information about the relative amounts of antigen in a system may be critically dependent upon the conditions used for comparison. It follows that results made obtained only under a single set of conditions might be misleading. How important these results are will depend on the frequency with which monoclonal antibodies with properties similar to X4 arise, but this cannot be sensibly estimated from the data from only three antibodies.

R.8 Estimation of the amount of antibody in hybridoma supernatants

R.8.1 Standardisation of solid phase assay: the need to measure antibody concentrations

The amount of monoclonal antibody secreted into the supernatants of hybridoma cell cultures can vary by a factor of more than 100 (unpublished observations; FAZEKAS de St.GROTH 1983). This is not simply because different hybridomas grow at different rates (the amount of antibody is proportional to cell number, unpublished observations), but also because of intrinsic differences in secretion rates (FAZEKAS de St.GROTH 1983). The absolute concentrations of antibody which are found in supernatants vary from approximately 1 to >100 µg/ml.

I have shown in sections R.7.3.1.1.2, R.7.3.1.2.4, and R.7.4.4.1 that non-specific binding by monoclonal antibodies in solid phase assays at high concentrations of antibody (10 - 100 µg/ml) can be so great as to swamp any specific effects. For this reason in solid phase assays, it is desirable to carry out assays using a standard concentration of about 1 µg/ml antibody. This applies especially to screening, where one would like to avoid false positives from the outset.

I should stress that this concentration guideline applies only to solid phase assays in microtitre plates. Immunological staining of cytological preparations, which can also be viewed (sensu lato) as a solid phase assay, may require higher concentrations, but the conditions of staining are very different from the conditions in microtitre plate wells. Incubation times are shorter, the surface topology of the antigen presenting surface very different and the volumes of wash solutions used per unit area of solid phase greater by a factor of 1000. In addition morphological cues can be used to establish the authenticity of specific staining of localised areas against a widespread low level of background staining.

The requirement for standardising the amount of antibody

raises the problem of how to estimate the amount of these proteins in cell culture supernatants containing at least a hundred fold excess of other proteins, present in the FCS added to to the medium to support cell growth.

Immunodiffusion assays are too insensitive: they cannot measure concentrations of antibody much below 50 µg/ml (my own observations; SPRINGER 1983). A variety of methods has been reported by other investigators. Classical RIA or competition ELISA in which the unknown supernatant competes with a labelled mouse antibody for binding to an antimouse antibody can be used and can be very sensitive (ENGVALL and PERLMANN 1971, SPRINGER 1983), but requires the preparation of labelled mouse antibody which would probably not be useful for any other purpose. A number of solid phase IRMA and ELISA methods have been reported (BOSWORTH et al., 1983, CRIPPS et al., 1983, LEMS-VAN KAN et al., 1983), but most of these are "sandwich" methods in which an anti-mouse antibody is first bound to solid phase, then the unknown and lastly labelled detector. These methods have three disadvantages. Firstly relatively large amounts of the first antibody (>1 µg/well) are required. Secondly the assay requires two antigen antibody incubations. Lastly, sandwich assays are subject to the "hook effect", whereby the assay response as a function of antigen concentration may be bell shaped, giving rise to ambiguous assay responses unless several dilutions of the unknown are used.

The method which I have developed avoids the above problems and has the advantage of simplicity. It uses only materials which are already in use in the laboratory (labelled anti-mouse antibody). It requires negligible amounts of hybridoma supernatant (< 1 µl) and requires only a single antibody-antigen reaction. The assay gives estimates of absolute concentration which appear to be correct within a factor 5 and is sufficiently sensitive to be able to measure antibody concentrations of between 1 µg/ml and 5 µg/ml in supernatants.

R.8.2 Properties of the standardisation assay

The assay simply involves diluting culture supernatant with buffer by a large factor (approx. 10^3), allowing the antibody in the diluted supernatant to bind to the solid phase and then measuring the amount of bound antibody with a labelled detector using either IRMA or ELISA techniques. The absolute amount of antibody is estimated by calibrating the assay with buffer dilutions of "pseudosupernatants", a series of known amounts of a mouse IgG standard (usually polyclonal mouse serum IgG) made up in cell culture medium (see M.2.5.2.3, p.63).

The dilution step is important to bring the assay response into the useful range. Figure 41a shows IRMA calibration curves obtained using "pseudosupernatants" diluted to different extents. As the inset to fig. 41a shows, the response of the assay to antibody concentration at all 3 dilutions is approximately linear (but see next section), but the sensitivity i.e. the slope of the gradient is greater at **higher** dilution.

The data from such calibrations is usually plotted in the form of a double logarithmic plot such as that shown in the main part of fig. 41a. In this kind of plot the linearity of the response is indicated by a line of slope 1. Other slopes indicate deviations from linearity (cf. next section R.8.3). The slope of the assay response in a **linear** plot is indicated in a log-log plot by the relative displacement of the lines along the ordinate axis. This form of plot is used to space out the measured data points so that may be more clearly seen, and to allow data from a wide range of calibration concentrations to be plotted. This point is illustrated by the inclusion of data points from a "500 µg/ml" pseudosupernatant in the log-log plot. Even on the small scale of the graph in the inset, on this scale these points would lie somewhere in the middle of fig. 41b. When such a wide range of antibody concentrations is used to calibrate the assay, transformation

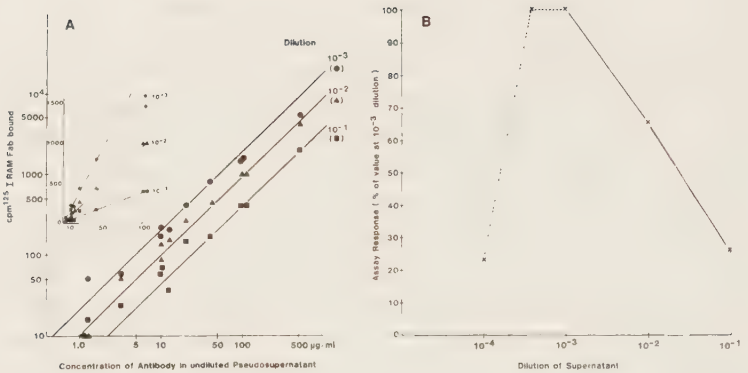

Fig. 41 **Standardisation Assay for Mouse IgG in hybridoma supernatants.**

Panel A **Double logarithmic plots of bound ^{125}IRAMFab as a function of antibody concentration and supernatant dilution.**

Pseudosupernatants (M.2.5.2.3) using either purified monoclonal antibodies S5/7/7/6, 32-5B6 (DREYER et al., 1981; a kind gift of C.DREYER), or purified mouse serum IgG (MILES). In this experiment no difference was detected between the behaviour of these antibodies so the data has been pooled. The assay was carried out according to M.2.5.2.3 except that 10^{-1} and 10^{-2} dilutions of supernatant were also used. Diluted supernatants were allowed to coat wells for 30 min at room temperature. All subsequent steps were on ice. Incubation with detector (1.25 +/- 0.01 -99% confidence limits- 10^5 cpm I^{125} RAMFab was for 17 hr. All points are means of duplicate values and have been corrected for non-specific background binding (culture medium alone 36 cpm +/- 17 cpm (95% confidence limits)). The difference between duplicate values was usually less than 20% of the mean. Error bars have been omitted for clarity. The calibration line has been fitted by inspection. The dilution corresponding to each of the lines and the symbols used to represent the data points belonging to each dilution are indicated on the right hand side of the panel.

Inset Same data (1 - 100 µg/ml range only) plotted on a linear scale. Symbols represent same data sets as in main part of figure.

Panel B **Expected enhancement of Standardisation Assay by "dilution effect"**

Composite graph obtained by combining the data from several experiments in which different combinations of dilutions were used.

of the data to logarithmic form is also necessary if data from lower antibody concentrations is to be meaningfully represented in the estimation of statistical parameters (otherwise the parameter estimates are overwhelmed by the

contribution from the most concentrated supernatants).

The low values obtained from the "100 µg/ml" and "500 µg/ml" pseudosupernatants at 10^{-3} dilution are almost certainly the result of experimental error. In many other experiments (this was a routinely used assay, cf. also fig. 42 in the next section), this effect was not seen. The assay does eventually saturate (IRMA technique) but only at antibody concentrations exceeding 1 mg/ml which are unlikely to be encountered in practice.

The absolute amounts of mouse IgG present in each well at a 10^{-3} dilution are very small in absolute terms. The amount of IgG present in 100 µl of a 10 µg/ml supernatant diluted to this extent is only 1 ng. Diffusion constraints and competition by serum proteins will render less than 10% of this amount able to bind to the well walls (see below), so that only 100 pg of IgG or 600 attomoles (= 600 10^{-18} moles = 4 10^{8} molecules) will finally be "seen" by detector. The minimum concentration of IgG in a supernatant which can be detected using this assay method is 0.5 - 1.0 µg/ml (see next section R.8.3). The sensitivity of the assay is therefore approaching the theoretical limits discussed in R.7.2.1.3 (cf. table 20) and suggests that RAM-Fab may contain a component (cf. R.7.3.2.2) with a K_{eq} for mouse IgG of 10^{11} or greater (?bivalent binding, R.7.2.2.4?).

McKEARN (1980) has also noted that it is possible to assay mouse antibody in this way in supernatants diluted 1/100.

Data from other experiments showed that the assay response was relatively insensitive to dilution over the range 1/500 to 1/2000, but fell sharply at higher dilutions. The expected dependence of assay response enhancement upon dilution ("dilution effect") is illustrated in fig. 41b. Note that this effect means that by **decreasing** the amount of a substance which is to be assayed the signal obtained from that substance increases not only in a compensatory fashion (such that the signal would remain constant) but also in absolute

terms. This concept is at first sight counterintuitive, but as I show below there is a rational explanation for at least part of the effect. I should point out that the increase in sensitivity which dilution affords is not very large, but the factor 4 - 5 which it provides makes the difference between being able to detect a supernatant containing 10 µg/ml unambiguously or confusing it with background.

The mechanism by which the dilution effect occurs is not fully understood. The principles propounded in the previous chapter (R.7), in their simplest form, do however predict that dilution should cause the assay response to remain unchanged as long as the amount of serum protein exceeds the binding capacity of the well wall.

The theoretically expected effect can be explained on the basis of the following facts. The amount of protein in the 20% FCS present in the supernatants is 7 mg/ml (Bradford Assay) and consists mainly of BSA (SDS-PAGE analysis, not shown). BSA has a diffusion coefficient somewhat greater than that of IgG (BSA, $D_{20,w}$=5.58 10^{-7} cm^2 s^{-1} - TANFORD (1961) - ; IgG $D_{20,w}$ = 4.3 10^{-7} cm^2 s^{-1} cf. M.7.5, p.86). To simplify the argument that follows I treat serum as if it only consisted of BSA. The protein binding capacity of a microtitre well surface filled with 100 µl liquid is assumed to be approximately 180 ng (see table 28, p.292). The amount of BSA present in a supernatant is present in vast excess over IgG; even at an IgG concentration of 100 µg/ml BSA is present in 160 fold molar excess.

The mechanism can then be understood as follows. If 100 µl of a 1/10 diluted supernatant is placed in a microtitre well it contains 7 10^4 ng BSA, or approximately 400x the amount of protein required to saturate the well surface. The amount of BSA necessary to saturate the well wall is contained in only 0.25% of the total volume of liquid in the well. This proportion of the well volume is present in a layer of liquid approximately 50 µm wide adjacent to the well wall. It will

only take about 1 s before the wall is saturated with protein. If the original concentration of IgG in the supernatant was 10 µg/ml, 100 ng will be present in the well. In 1 s the maximum proportion of IgG which could bind to the wall, even if no competing protein were present, is 0.2% or 200 pg. In fact there is competition, BSA will occupy a fraction of the potentially available sites and make them inaccessible. However, because the BSA:IgG ratio is constant, the correction to the potential IgG binding capacity estimate which this consideration requires should be the same for all dilutions, provided that the amount of protein in the well is greater than that required to saturate it.

If a 1/10 diluted supernatant is diluted by a further factor 10 and used for assay, the amounts of BSA and IgG in the well will decrease to 7 10^3 ng and 10 ng respectively. The minimum amount of time (neglecting the decrease in binding rate as the wall becomes saturated with protein) required by BSA to diffuse to the well walls before they become saturated will be approximately 15 s (at room temperature). The maximum proportion of IgG which can diffuse to the walls in this period of time is 2.0% of the total (=200 pg). Thus, although there is 10 fold less IgG present in the well, it binds approximately 10 fold more efficiently, with the end result that the same absolute amount binds using either concentration.

The argument repeats for a further factor 10 dilution (10^{-3} dilution), except that the time available for binding is now >20 min which is only a little less than the time which is used to coat the the wells with protein anyway. Further dilution by a factor 10 causes the total amount of protein in the well (70 ng), to fall below that required for saturation 180 ng. Thus, potentially, all IgG at 10^{-4} dilution (100 pg) would be able to bind, the correction for binding site exclusion by BSA during this period would be negligible, but because of the kinetics of binding the operator would require to allow a very much longer period of time than that required

for the normal standardisation assay. In 30 min at room temperature the maximum proportion of IgG which can bind is 25% (25 pg). The observed decrease in assay response between a 10^{-3} dilution and a 10^{-4} dilution is a factor 5, which suggests that a maximum of 125 pg of IgG actually binds compared to the theoretical maximum of approximately 200 pg. This suggests that the binding site exclusion correction at lower dilutions should be on the order of a factor 2.

The above mechanism only predicts that the assay response should remain constant. In fact it increases with dilution. Control experiments (not shown) demonstrate that the amounts of IgG bound in the presence of 10^{-4} diluted serum are only 30% less than those bound when pure IgG is used to coat the wells, in approximate agreement with the above hypothesis. This means that the explanation for the "dilution effect" must be sought in a mechanism which **decreases** the rate of diffusion or binding of IgG to the well surface at high serum concentrations (low dilutions). Furthermore this effect must work relative to any effect upon BSA. It is not difficult to imagine candidate mechanisms. At the lowest dilutions of supernatant the protein concentration is so high that it may cause the hydrodynamic behaviour of protein molecules to depart significantly from ideality. For instance a protein-protein interaction might cause the formation of short-lived complexes which would reduce the effective diffusion coefficient of IgG relative to that of BSA. Effects of salt or pH on the retention of initially bound IgG, similar to those reported by McKEARN (1980) might also play a role.

In summary, the "dilution effect" can be exploited to provide a simple and sensitive assay for IgG in the presence of a complex mixture of proteins. A consideration of solid phase system properties shows that it is possible to provide a plausible partial explanation for the effect which at first sight seems counterintuitive.

R.8.3 Standardisation measurements: a measure of absolute concentration

The assay described in section R.8.2 gives a standardised measure of the amount of antibody in a cell culture supernatant. For antibodies other than those used to calibrate the assay, the units of measurement are "µg equivalents/ml of X" where X is the calibration antibody. This raises a question. Are "µg equivalents/ml" identical with µg/ml in absolute terms? The data in Fig. 42 show that the answer to this question must be an unequivocal "no".

The assay response of the 3 purified antibodies used to calibrate the IRMA which are shown in fig. 42a differed by as much as a factor 4 when identical amounts of antibody were assayed. When ELISA was used, the largest difference which was found was even greater, a factor 10, as shown in fig 42b.

The data presented in figure 42 bears witness to the sensitivity and reliability of both assay methods. Note that the ELISA assay would be curvilinear if plotted on a linear scale (see figure legend), the reason for this is not known, but saturating amounts of detector are used in ELISA in contrast to near limiting amounts in IRMA. However, logarithmic transformation and the use of a double logarithmic plot allows the dependence between the two assay variables and the variation in this dependence to be satisfactorily parameterised using linear regression methods.

For either assay most of the data obtained using supernatants containing more than 6.25 µg/ml antibody corresponds very satisfactorily to the predicted fit. 6 µg/ml can therefore be considered as the minimum concentration of antibody that these assays can reliably and universally detect. However concentrations of less than 1 µg/ml are clearly detectable from the antibody giving the most sensitive assay response (P11).

The scatter of most of the individual data points on the T7 ELISA line (fig. 42b) is large, but this is itself a result

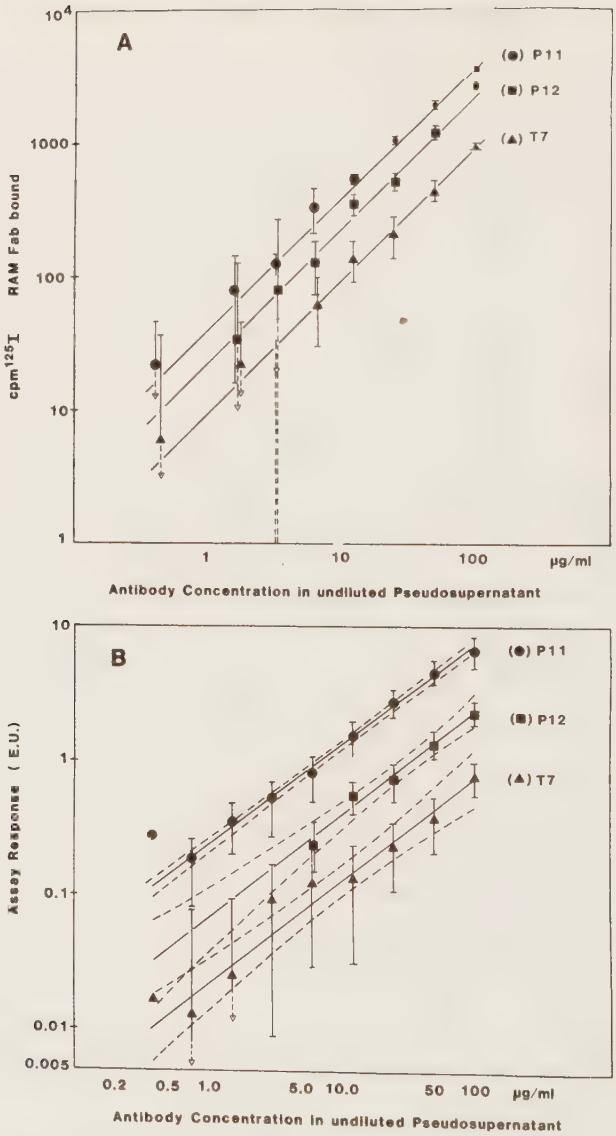

Fig. 42 (see next page for legend)

Fig. 42 **Standardisation assay calibration curves obtained from different monoclonal antibodies are different**

The same three purified antibodies P11/3/6 (P11), T7/3/3/4 (T7) and P12/8/6 (P12) were used to calibrate the two standardisation assays shown here. The lines corresponding to each antibody and the symbols used to represent the data obtained from each are indicated on the right hand side of each panel below.

Panel A **IRMA**

Double logarithmic plot of regressions obtained from untransformed (i.e. linear) data. A better fit would be obtained by plotting:

$$\text{bound cpm} = X\,[\text{supernatant conc.}]^{0.9},$$

where X is a multiplier characteristic for each antibody, but the deviation from linearity is not statistically significant. The solid symbols and error bars show the means and 95% confidence limits of quadruplicate determinations corrected for non-specific binding (cell culture medium, 25 cpm +/- 28 cpm - 95% confidence limits). The data points below 6.25 µg/ml which may not be significantly different from zero are marked by the dotted error bars. Using the logarithmic fit noted above, and the data from > 6.25 µg/ml the difference in displacements of the calibration lines (=assay sensitivity) tested using the t statistic was statistically significant at P<5% (8 d.f) for the P12,P11 and P12,T7 differences, and at P<1% (8 d.f) for the P11,T7 difference.

Panel B **ELISA**

Double logarithmic plot of dependence between E.U. response and pseudosupernatant antibody concentration. The regression fitted to log transformed data by the method of least squares is indicated by the solid lines. In a linear plot these regressions would be equivalent to curves of the form:

$$\text{bound cpm} = X\,[\text{supernatant conc.}]^{0.78},$$

where X is the parameter describing the assay response sensitivity of each antibody. The dashed curves on either side of the regression indicate the 95% confidence limits of the E.U. values predicted from the regression parameters. The filled symbols and error bars represent the means and 95% confidence limits of octuplicate measurement of ELISA response corrected for non-specific response (from cell culture medium alone =0.184 (T7), 0.178 (P11), 0.205 (P12), the 95% confidence limits +/- 0.05 in each case) which were actually measured. Dashed lines indicate an error distribution overlapping with zero. The P12 measurements below 6.25 µg/ml gave nonsensical values and were rejected, as a result the P12 regression data is weighted, which explains the greater divergence of the E.U. estimate confidence limits at low supernatant concentration values. Using the t statistic, the difference in displacement along the ordinate axis (X above) was statistically significant at P<5% for P11 and P12 (10 d.f.) and at P<1% for the other two pairs of lines (10 d.f and 13 d.f).

of the low response which this antibody gives. The specific assay response of the five lowermost data points have values which are less than twice the non-specific binding response (0.184 E.U). Despite the large scatter associated with the individual data points the means of the T7 estimates allow the estimation of a satisfactory set of regression error parameters (dashed curves in fig. 42).

For the sake of clarity, only a selection of data is shown in fig 42. A panel of 3 other monoclonals and mouse serum IgG was tested in parallel in the same experiment. The results, in terms of the relative sensitivity of the assay response of each of the antibodies relative to mouse serum IgG are summarised in table 38.

Monoclonal Antibody	Relative Sensitivity in ELISA	IRMA
P11/6/3	1.69	0.98
Mouse Serum IgG (**STANDARD**)	1.00	1.00
P12/8/6	0.52	0.70
P3-X63 (MOPC21)	0.52	0.49
Z4/10/9	0.52	0.36
X4/8/6	0.17	0.36
S5/7/7/6	0.17	0.31
T7/3/3/4	0.17	0.25

Table 38 **Relative signal in Standardisation Assay given by pseudo-supernatants containing equal (spectrophotometrically determined) concentrations of antibody**

There is not necessarily any contradiction between the low sensitivity of the S5 antibody relative to mouse IgG in this experiment and the similar sensitvity between these antibodies reported in fig. 41. The experiments were carried out a year apart, and used used different batches of mouse serum IgG (the preparation used for the experiment shown in fig. 42

contained an additional minor band, presumably a proteolytic breakdown product, on SDS-PAGE analysis, and this served as a marker to distinguish the two batches). A different composition of the two IgG preparations (e.g. a different distribution of IgG subclasses, not the proteolysis product, which was very minor) may well account for the difference. I was unable to test this.

Two types of difference are observable in table 38:

1) Differences in response by the same antibody using different assay procedures. Both P11 and Z4 give relatively greater responses in the ELISA compared to IRMA.

2) Differences between assay response in the same assay procedure. Using either technique the sensitivity of individual monoclonal antibodies appears to be clustered into three groups.

Response less than 50% Mouse IgG:	T7, X4, S5
Response between 50% and 100% Mouse IgG:	P12, P3
Response equal to or greater than Mouse IgG:	P11

In considering the first effect, it should be noted that both the assays from which results are shown in table 38 were carried out in the same type of PVC plate. This leaves the difference in detector antibody as almost the only possible cause of the difference between the two assays. An antibody directed against the whole mouse IgG molecule was used for the ELISA and one only directed against the Fab part of the molecule was used in IRMA. The ELISA antibody may have contained a larger proportion of antibodies directed againt determinants present on the P11 and Z4 IgGs (see R.7.4.6). SPRINGER (1983) has reported that antisera raised against whole mouse IgG often contain high titres of activity against subclass specific determinants and that the proportion of the activity directed against a particular subclass is often strongly biased. Consistent with this suspicion is the observation that P11 is the only IgG2a subclass antibody.

This hypothesis could be tested using a panel of IgG2 antibodies. SPRINGER (1983) recommends the use of anti-Fab antibodies to avoid such effects and the reduced range of variation in IRMA is consistent with this concept.

Unfortunately not all anti-Fab preparations cause the same decrease in variation. In another series of experiments (data not shown) a pair of phosphatase labelled anti-(whole molecule) and anti-Fab antibodies obtained from another company (Jackson Immunoresearch), showed a similar spread in their range of activity when tested against a panel of monoclonals. In a single case the results from this pair of antibodies also provided evidence for the thesis that variation in antibody specifity can be a major cause of variation in assay response. The antibody Q18/5/4 (an IgG2a) gave the highest relative response with the anti-Fab preparation and the lowest relative response with other preparation.

The second question of what the causes of variation in assay response in the same assay may be is in part answered by the above observations. Anti-antibody preparations bind to particular monoclonal antibodies unequally, and different anti-antibody preparations differ in their preferences. The differences might either reflect a difference in the number of antigenic determinants recognised per mouse IgG molecule or the proportion of anti-antibody directed against unique determinants. The affinity of the antibodies recognising different determinants may also play a role. I showed in section R.7.4.5 in IRMA (albeit not one where IgG is bound directly to solid phase) P11 is bound substoichiometrically, yet P11 gives one of the highest responses. This suggests that, at least in IRMA, many solid phase bound IgG molecules may go undetected because the concentration of anti-antibody directed against a small number of antigenic determinants is insufficiently great to allow efficient binding (cf. R.7.2).

Selective binding (probably more accurately retention cf. R.7.3.5) to the solid phase, may also contribute to differences in assay response. I have no evidence which bears on this point, but there is a report (McKEARN, 1980), that the efficiency with which different monoclonal antibodies bind to PVC varies. In addition this author has presented data to show that the binding (?retention) of mouse IgG in 1/100 diluted supernatants can vary by as much as a factor 10, depending on the pH and salt concentration conditions in which absorbtion takes place. He reports that the optimum binding conditions vary between antibodies but unfortunately presents no data on this.

I have no data which allows a direct estimate of the relative contibution of these two mechanisms to the variation in assay response which I report here. It is however of interest that in assays either of Drosophila nuclei or of hnRNP extracts that the signal obtained in IRMA from S5 and X4 **antigen** was consistently at least 5 - 10 fold lower than from P11 **antigen**, although RISAU (1983) has estimated that the amounts of both antigens in the nucleus is at least as great as that of P11. If RAMFab were to recognise bound X4 and S5 **antibody** in these asays with the same low efficiency relative to P11 as in the standardisation assay, this could explain at least a part (30% - 100%, given the range of RISAU's estimates) of the discrepancy

To summarise, the standardisation assay which I have described does not allow the concentration of mouse IgG present in hybridoma supernatants to be measured precisely in absolute terms. It does however give a standardised value, which guarantees that the same amount of a particular monoclonal antibody can always be used for a particular test. Thus, once the optimal conditions for a procedure using that antibody have been developed they can be reproduced, regardless of the concentration of antibody in the supernatants which are available, and without having to purify

the antibody. The non-specific binding effects in assays for antigens referred to in the first section of this chapter can be thus be avoided. There is evidence for two effects which contribute to the variation in standardisation assay response: differential recognition by anti-antibody and differential adsorption to solid phase. In principle, it would be possible to control for both these effects (for instance by testing each monoclonal with a battery of anti-antibodies), but this would eliminate the simplicity of the assay, which is one of its advantages. A more efficient strategy is to employ the assay in its simplest form, but to be aware of its limitations. Some improvements should however be made. A monoclonal antibody giving an assay response in the middle of the sensitivity range rather than mouse IgG should be used as a calibration standard. This will eliminate problems caused by variation in the composition of different batches of serum IgG. In addition, by using a mid-range value, the standardised units will approximate absolute units more nearly (e.g. by using P3 as the standard for IRMA the difference between µg equivalents of P3 and µg/ml in absolute units would be no more than a factor 2). On a long term basis, it would be desirable to produce a defined anti-mouse antibody mixture, using rat anti-mouse monoclonals directed against the most common mouse IgG determinants, to allow precise immunological quantitation.

RESULTS and DISCUSSION

PART II

APPLICATIONS OF IMMUNOLOGICAL METHODS

PART IIA

Quantitative Estimates of Drosophila Antigens

R.9 Frequency of hnRNP-associated hybridoma defined antigens in Drosophila Kc cell nuclei

In this chapter, I make gross estimates of the frequency of hnRNP-associated polypeptides based on the hnRNA and hnRNP protein content of the Kc cell nucleus. These frequencies are compared with those of hnRNP-associated non-packaging antigens (RISAU et al., 1983) which can be calculated from solid phase assay.

R.9.1. Composition and fractionation of Drosophila Kc cells

There is a dearth of information about the macromolecular composition of insect cell nuclei. Data is available for vertebrate cell nuclei (see table 52, R.9.2.1.1), but since the DNA content of one of these nuclei is about 10 times that of an insect cell nucleus, it would seem reasonable to suspect that there might be some differences. This suspicion appears to be correct (R.9.2.1.1). The results detailed in section R.9.1 are important because they remedy this deficiency. It was necessary to have quantitative information about nuclear composition to be able to estimate the purity of the hnRNP fraction in which a number of monoclonal antibody defined antigens were found (RISAU et al., 1983). Section R.9.1.1 describes how this fraction was obtained. The information also allows quantitative estimates of antigen frequency (R.9.2) and shows that the Kc cell line used in our laboratories is no longer diploid.

Because there is no comparable data and because cell lines maintained in culture can vary (see R.9.1.5), I have carefully documented the statistics of these cells and the uncertainties associated with them. This should allow other workers to judge how their cell lines compare with ours.

R.9.1.1 Fractionation scheme

Fractions obtained from various steps of the scheme shown in fig. 43 were analysed. This scheme was devised by W.RISAU for obtaining hnRNPs (an abbreviation for heterogeous nuclear RNA containing Particles i.e particulate material containing precursors of mRNA), for full details see RISAU (1983). The composition of the nuclei is discussed in section R.9.2.1. The high value of RNA to DNA ratio in the fractions obtained after sedimentation velocity separation indicates that this method achieves a highly selective extraction of RNA containing particles. Note, however that recovery of RNA from sucrose gradients is only 60%. The following sections detail the reliability of the estimates.

R.9.1.2 A remark about statistical procedures - "worst case" estimates

Many of the determinations of amounts of components which are described in section R.9.1 are expressed in normalised form

i.e. units per cell.

The number of cells (or nuclei) and the measure of a particular component were determined on independent samples (often of greatly different size) taken from the same preparation. By taking multiple samples it was possible to estimate the variation associated with each parameter.

One might naively imagine that being able to estimate accurately the variation of both dividend and divisor would also allow a precise estimate of the variation of the quotient (i.e. the normalised values), but this is not the case. The reason why such an estimate is not easily obtainable is non-trivial, since it involves the solution of a statistical

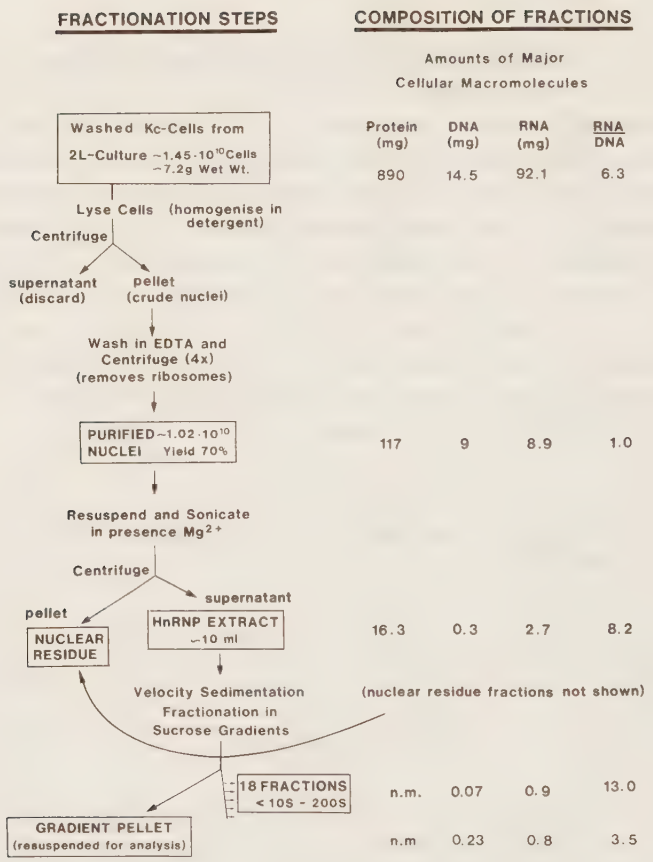

Fig. 43 **Summary Diagram of a fractionation of Drosophila Kc cells from which hnRNPs were obtained and of the macromolecular composition of the component fractions.**

Fractionation was carried out by W.RISAU who kindly provided aliquots of the fractions for analysis. In the panel on the left the steps involved in the fractionation are briefly outlined. The fractions whose names are boxed in were analysed for their macromolecular composition, which is shown in the right hand panel. n.m. = not measured.

problem for which there is as yet no analytical solution (G.WAGNER personal communication, G.DEICHSEL personal communication).

Further details of the problem and a possible empirical way to obtain an exact solution to the problem are described in more detail in section M.7.2. The suggested solution has not however been attempted because of the inordinate amount of time and computing effort it would have required

In view of these difficulties, a gross estimate of the variation of the normalised values is provided by making "worst case" estimates. These estimates simply involve taking the upper and lower limits of the estimates of each of the determinations at a particular level of probability, such as to give rise to the most divergent estimates of the quotient. The mathematical complications mentioned above preclude a precise estimate of the probability of these "worst case" values. Intuitively, however, one would expect it to be lower than the probability of either of the individual determinations. It follows that where, for instance, the 95% confidence limit values of each of the determinations have been taken, the probability that the true mean value is lower than the "worst case" lower limit will be under 2.5%, but just how far under 2.5% cannot be calculated.

R.9.1.3 Cell number estimates and wet weight of cells

The result of a count on one batch of cells and of counts on two different batches of nuclei are presented in table 39.

Notice that for the cells there is less than 1% probability that the true value deviates from the estimate obtained here by more than 10%. This is also true for batch B of nuclei. However, with batch A nuclei the probability that a deviation of this extent could occur is approximately 10% (i.e. this estimate is approx. 10 fold less precise than the others). The cause of this uncertainty is the clumping of

nuclei which often occurs. Although this clumping is not serious enough to prevent handling of the nuclei and is not even immediately obvious on microscopic inspection, it does give rise to a greater variation in the actual numbers of particles counted in each haemocytometer field, and hence generates a greater degree of uncertainty in the estimates.

Batch	Sample Nr.	Fields counted	Cell Nr. (Cells/ml)	S.E.M	Confidence limits 90%	99%
			CELLS			
			(all values in last 4 columns x 10^{-6})			
A	1	(10)	7.43	0.21		
	2	(10)	7.06	0.19		
	pooled data	(20)	7.25	0.15	6.99 - 7.50	6.82 - 7.68
			NUCLEI			
			(all values in last 4 columns x10^{-8})			
A	1	(8)	7.81	0.61		
	2	(8)	8.19	0.54		
	pooled data	(16)	8.00	0.40	7.30 - 8.70	6.82 - 9.18
B	1	(8)	7.53	0.04		
	2	(8)	7.73	0.03		
	pooled data	(16)	7.63	0.025	7.10 - 8.20	6.89 - 8.37

Table 39 **Particle counts of cell and nuclei suspensions used for biochemical analysis.**
2 independent samples (separate pipettings) were taken from each batch of cells or nuclei and suitably diluted to bring the particles they contained into the countable range. 8 or 10 1.0 mm^2 (cells) or 0.04 mm^2 (nuclei) haemocytometer fields were counted for each sample. For counting protocol see M.7.1.

- -

The nuclear preparations also contained a small amount (<10% in terms of frequency) of debris which, using phase contrast microscopy, could be distinguished from nuclei on the basis of size. To confirm that I was able to discriminate

accurately between nuclear and non-nuclear material, nuclei were counted after fluorescence staining with a small amount (approx. 10^{-6}M) of ethidium bromide (debris did not stain). There was no statistically significant difference ($P<20\%$) between the two counts (data not shown). Counting of fluorescent nuclei was judged to be more convenient since it did not require having to discriminate between two sets of objects and was therefore used routinely.

The wet weight of cells was estimated using two separate aliquots of batch A. To reduce intercellular fluid to a minimum, the cells were compacted by centrifugation at 9,000 rpm in a Sorvall HB4 rotor (g_{max} = 13,000) for 5 minutes, and the centrifuge tube in which the cells were pelleted was carefully wiped free of any excess moisture before the cells were weighed. The results are shown in Table 40.

	Nr. Cells ($\times 10^{-8}$)	weight of pellet (mg)	mean weight per cell (pg)	"worst case" limits (uncorrected for intercellular water)
Sample 1	1.800	92	511	
Sample 2	1.895	101	533	
Pooled data	3.68	193	524	493 - 555

Table 40 **Wet weight of Drosophila Kc cells**
In calculating the "worst case" limits, the weighing error has been assumed to be negligible and the 99% confidence limits of the cell number estimate have been used.

While the error of the weight measurement itself can be considered negligible, the amount of intercellular water is unknown and provides a major source of uncertainty. Assuming that the trapped intercellular liquid made up 5% of the weight of the pellet (a mid-range value of the corrections applied when determining erythrocyte volumes by centrifugation [cf. ALTMAN and DITTMER (1964) pp.263 - 264] and that this figure is unlikely to be in error by more than a factor of 2, then this would give an estimate for the wet weight of a Drosophila Kc cell of:

498 pg

with "worst case" bounds of 444 pg (10% H_2O and the lower 1% probability bound for cell number) and 541 pg (2.5% H_2O and upper 1% probability bound for cell number). The 498 pg value will be assumed in all subsequent calculations concerning the wet weight of the cells.

Assuming a cell approximates a perfect sphere with density 1.0, a mean value for the diameter of the cells of 9.8 µm was calculated. This value may be compared with an arithmetic mean diameter of 7.5 µm (range 5 - 14 µm; wet weight of cell with 7.5 µm diameter = 220pg) for Drosophila Schneider line cells growing in serum free medium (LENGYEL et al., 1975).

R.9.1.4 Protein content of Drosophila Kc cells

The protein content of cells and various subcellular fractions from cell batch A was estimated by the Biuret method and is tabulated in table 41 below.

Unfortunately the range of the nuclear protein determination is very large. This coupled with the small number of measurements and the uncertainty of the nucleus count (see table 39) means that there is a 1 in 5 probability, on the basis of these measurements, that the estimated figure could be in error by a factor as great as 2. However, the sum of the protein present in the hnRNP extract and the nuclear residue should be approximately equal to the amount in the nucleus (slightly less because the nuclei are washed prior to preparation of the hnRNP extract) and allows an independent estimate of the amount of nuclear protein. The value of the sum is 11.7pg with 90% confidence limits 9.5 - 12.3 (assuming a maximum of 50% error in the measurement of the hnRNP extract and the confidence limits shown in table 41). These figures

Fraction	mean mass / cell (or cell equivalent)	number of measurements (n)	range (or 95% confidence limits for n >2)
	pg		
Cells	61.1	2	±1.15pg
Nuclei	11.3	2	±1.45pg
hnRNP	1.6	1	-
Nuclear Residue	10.1 (8.7 - 11.5)[1]	3	±0.52

[1] Worst case limits taking 90% confidence limit values of protein and cell number determinations.

Table 41 **Protein content of Drosophila Kc cell fractions**

suggest that the measured value for nuclear protein is substantially correct.

A summary of the relative amount of protein in the various fractions is provided in table 42 below.

The percentage of the cell wet weight occurring as protein compares with figures of 12.6% and 12.9%, reported for mouse and rat liver respectively (cited in ALTMAN and DITTMER 1964), and would therefore not appear to deviate greatly from values found in mammalian cells.

Little significance can be attached to the proportion of total cell protein found in the nucleus, because of the extreme variation in the proportion of the cell volume occupied by the cell nucleus (e.g. << 5% for cells such as oocytes to > 50% for some kinds of lymphocytes). Drosophila Kc cells clearly belong to the more "nucleus-rich" end of this distribution, making them an efficient source of this organelle.

Fraction	% wet weight	% cell protein	% nuclear protein measured	% nuclear protein calculated
Total Cell	12.3	100	-	-
Nuclear (direct)	2.3	18.5	100	
(calculated)	2.3	15.8		100
hnRNP Extract	0.3	2.6	14.1	17.9
Nuclear Residue	2.0	16.5	89.4	82.4

Table 42 **Summary of the relative proportions of protein in various Kc cell fractions.**
Two values are given for nuclear protein: "measured" refers to the value in table 41 and "calculated" to the sum of hnRNP extract and nuclear residue.

- -

R.9.1.5 DNA content of Drosophila Kc cells

Table 43 shows the data gathered from 3 separate determinations of DNA. Two of these determinations (1 and 2) were made using the DABA method and one (Nr. 3) using the ethidium bromide (EtBr) method (see section M.1.2) The reliability of the various estimates is not identical. In determination Nr.1 the calibration curve was obtained from 5 single data points and fitted by inspection: no attempt was made to ascertain the precision of the fit. In determination 2 a linear regression of the values of fluorescence intensity on the amount of DNA in the calibration standards was fitted using the method of least squares. The fit was extremely good, as fig. 44 shows. The estimates from determination Nr. 2 are therefore the most precise of the three. There is also good reason to to regard them as the most reliable: this conclusion is based upon the ability of the DABA method to correctly estimate the amount of DNA in the chick erythrocytes used as external standards. The observed value of 2.52 pg per cell (table 43, last line) agrees well with the value of 2.50 pg found by RASCH et al., (1971) using the Feulgen method and microdensitometry. It may

Fig. 44 **Calibration curve for the determination of DNA using the DABA method.**
The solid line is the regression fitted by the method of least squares. The dashed lines indicate the 95% confidence limits for the regression. The data points are the means of duplicate or multiple values. The vertical error bars show the range (duplicate values) or 90% confidence limits (multiple determinations).The horizontal error bars indicate the errors which would occur with a pipetting error of ±0.5 µl below 10 µl and of ± 1.0 µl in the range 10-100 µl.

also be compared with four other literature values ranging between 2.2 and 2.61 pg (mean 2.36 pg) which are cited by VENDRELY (1955). The imprecision of this estimate is less satisfactory (the worst case estimates show 20% deviation from the mean), but this is to a large extent the result of imprecision in the estimate of the cell number (90% confidence limits deviate 8% from the mean).

The reliability and accuracy of data from determination Nr.3 was much less satisfactory. The source of this inaccuracy lay in the least squares fit of the calibration regression,

Fraction	Determination Nr.	Cell Batch	Amount DNA measured (µg)	Concentration DNA in original fraction. (µg/ml)	DNA per cell or cell equivalent. (pg)
Cells	1	A	3.44 (2.96-3.97)	30.6[1] (26.8-35.4)	0.84 (0.71-1.01)
"	2[2]	A	1.23 (1.16-1.29)	40.8[1] (38.6-43.0)	1.13 (1.03-1.23)
"	3	A	3.47 (3.30-3.63)	34.7[1] (33.0-36.3)	0.96 (0.88-1.04)
Nuclei	1	A	4.30 (no varn.)	860 (no varn.)	1.08 (0.99-1.18)
"	2[2]	B	1.26 (1.20-1.32)	630 (600-660)	0.83 (0.75-0.92)
"	3	B	11.4 (10.8-12.1)	572 (541-603)	0.75 (0.66-0.85)
RNP extract:	1	A	0.84 (0.82-0.86)	24.0 (23.4-24.6)	0.03 (0.027-0.033)
"	2[2]	B	0.75 (0.66-0.84)	25.0 (22.0-28.0)	0.032 (0.027-0.039)
"	3	B	0.60 (0.44-0.76)	30.0 (22.0-38.0)	0.038 (0.027-0.049)
Nuclear Residue	**INCONSISTENT RESULTS**				
hnRNP gradient pellet	1	A	NOT MEASURED		
"	2[2]	B	0.79 (0.70-0.88)	15.8 (14.0-17.6)	0.021 (0.018-0.023)
"	3	B	0.93 (0.78-1.08)	18.6 (17.1-20.2)	0.024 (0.020-0.028)

continued from p.378

Fraction	Determination Nr.	Cell Batch	Amount DNA measured (µg)	Concentration DNA in original fraction. (µg/ml)	DNA per cell or cell equivalent. (pg)
Sum of all fractions from Sucrose Gradients (=recovery non-pelletable DNA in hnRNP extract)[3]	1	A	NOT MEASURED		
	2	B	0.53 (0.29 - 0.78)	n.a. (0 - 0.65)	0.003 (0.002-0.005)
	3	B	0.99 (0 - 2.5)	n.a. (0.3-1.9)	0.012 (0 - 0.031)
External Standard (Chick Erythrocytes)	2	-	between 0.15 µg & 8.4 µg (different concentrations)	2.52 (2.27-2.75) at 10^6 cells/ml	2.52 (2.1-3.0)

Notes [1] The cells whose particle count is given in table 39 were concentrated 5 fold before DNA estimation.

[2] Fourfold replication (most other values means of duplicate observations).

[3] The actual concentration range found in the fractions rather than the 95% confidence limits is given in column 5 ("Concentration").

Table 43 **Estimations of the amount of DNA in Drosophila Kc cells and Kc cell fractions.**

Data from 3 independent determinations are shown. In determinations Nr.1 and 2 the DABA method and in Nr. 3 the EtBr method was used. Column 3 shows the actual amount of DNA in the aliquot whose value was determined. In columns 3 and 4 95% confidence limits for the mean value of the estimate are shown in brackets (see M.7.3). In the last column the limits are worst case estimates using the limits shown in columns 3 and 4 and the 90% limits of the particle counts shown in table 39.

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fig. 45, which did not pass through the fluorescence blank value, leading to the unlikely prediction that 0.24 µg DNA should give zero fluorescence enhancement and the nonsensical result that the amount of material recovered from the sucrose gradient exceeded the amount applied to it. Several explanations for this state of affairs can be imagined:

Fig. 45 Calibration curve for the determination of DNA using the Ethidium method.

The line of alternating dashes and dots is the regression used to estimate the amounts of DNA. The solid line is the regression fitted by the method of least squares. The dashed lines indicate the 95% confidence limits for the regression. The data points are the means of duplicate or multiple values. The vertical error bars show the range (duplicate values) or 90% confidence limits (multiple determinations).The horizontal error bars indicate the errors which would occur with a pipetting error of ±0.5 µl below 10 µl and of ± 1.0 µl in the range 10-100 µl.

a)The calibration curve data is insufficiently precise, as a result of which there is sufficient variation in the data to allow a fit of this sort to occur by chance.

b)there could be a genuine non-linearity in the dependence of fluorescence enhancement upon the amount of DNA for small amounts of DNA.

c)there could be a systematic error in the determination of the DNA fluorescence enhancement values (i.e the slope of the calibration curve is correct but the intercept with the x

axis is incorrect).

Empirically it was found that simply correcting the estimates of the DNA content of the unknown samples giving the lowest fluorescence enhancement values (hnRNP extract and the sucrose gradient fractions) by the extent of the fluorescence blank "error" gave rise to reasonable agreement between the estimates provided by this method and by the DABA method in all fractions except those obtained from the sucrose gradient.

This correction is formally equivalent to assuming case c) above. There is no way to test whether this is the correct explanation, but it is possible to test the probability that such a "systematic error" might have occured as a result of the variation in the data itself (explanation a). As fig. 45 shows, such a calibration curve would fall just outside the 95% confidence limits. (i.e. if the calibration curve which passes through the fluorescence blank value is correct and were the calibration to be repeated many times, with a similar variation in the data to that found here, a calibration curve such as the one found here would expected to occur by chance once in every 40 calibrations). In the absence of any independent data, rejection of a best fit regression as the appropriate form of describing the data would be difficult to justify. However, since there is good reason to have confidence in the DABA data (see above), and since the "best fit" does not lead to the calibration passing through the fluorescence blank value as expected, there is a case for assuming a less probable fit to the data. The estimates shown in table 43 have been obtained from this less probable calibration curve. This interpretation of the data only causes noteworthy alteration of estimates of the amount of DNA in samples containing small amounts of this substance. An alteration of less than 8% is caused in determinations carried out on samples containing more than 3 µg DNA.

Whether or not manipulation of the data in this manner is justified, the source of error (or imprecision) appears to be

specific to the determination of the extent of fluorescence enhancement by DNA. No such problem was encountered in determining the amount of RNA by its fluorescence enhancement (see Section R.9.1.6).

It was not possible to measure the DNA content of the nuclear residue with either of the methods. The residue material was compacted by sedimentation and in addition was somewhat viscous. Attempts to resuspend it in a homogeneous form (including extensive sonication) were not very satisfactory. At any rate attempts to measure the DNA content of this material gave rise to uninterpretable results. This observation is not in contradiction to the ability to measure the protein content of this material, since different sample sizes were involved and it was possible to widen the pipette tips (to avoid clogging them) for the protein determination. This ploy did not succeed for the very much smaller amounts required for the DNA determinations. Possibly cutting off the apices of the pipette tips in order to widen them introduced too much error.

Comparison of the estimates of the amount of DNA found in cells and nuclei by the two different methods shows that less is detected using the ethidium bromide (EtBr) method: a conclusion which is insensitive to how the calibration curve data is interpreted. There is reason to suspect (see Section R.9.1.6) that this difference might result from DNAase action during the preparation of the samples for the EtBr method. The amounts shown by the EtBr method in table 43 are therefore probably lower limits. In arriving at a "most likely" estimate of the amount of DNA in the various fractions of Kc cells, summarised in table 44 (based on the pooled data from all 3 determinations), the EtBr estimate has been corrected for putative DNAse action. This does not alter the estimate of the amount of DNA in Drosophila Kc cells by more than 6%.

Cell Fraction	Absolute amount of DNA/cell (pg)	% total cell DNA	% nuclear DNA	% hnRNP Extract DNA
Cells	1.03[1]	100	-	-
Nuclei	0.91[1]	88.3	100	-
hnRNP Extract	0.033	3.62	3.3	100
Nuclear residue (calculated)	0.88	85.4	96.7	-
hnRNP gradient pellet[2]	0.023	2.2	2.5	70
hnRNP sucrose gradient fractions (calculated)[2]	0.010	1.1	1.2	33
(actual recovery)	0.007	0.7	0.8	21

[1]EtBr contribution to pooled data corrected for putative effect DNase action
[2]The amount of the hnRNP extract analysed in each sucrose gradient was obtained from approx 7.5 10^8 cells (approx. 100 - 200 ml of the original cell culture) and contained 25 µg DNA.

Table 44 **Summary of the expected amounts and proportions of DNA present in Drosophila Kc cells and cell fractions.**
The figures here are the "most likely" estimates and it is not possible to calculate a meaningful estimate of their precision. However for the reasons explained in the text it is thought that it is highly unlikely that any of the absolute estimates is error by a factor greater than 50%. The values were obtained by pooling all data.

Even allowing for the limitations caused by the minor discrepancies in the data discussed above, the following five major conclusions may be drawn about the distribution of DNA in Drosophila Kc cells and cell fractions:

1) All 6 independent estimates of the amount of DNA in Drosophila Kc cells or cell nuclei give values exceeding 0.75 pg. This value is more than double the diploid amount of DNA expected for Drosophila cells (0.36 pg, RASCH et al., 1971).

2) less than 5% of the nuclear DNA is contained in the hnRNP extract.

3) at least 50% of the DNA in the hnRNP extract is pelleted by high speed centrifugation in sucrose gradients.

4) less than 2% of the total nuclear DNA is present in sucrose gradient fractions which contain hnRNP. The maximum amount of DNA detectable in any one of the 1.2 ml fractions analysed here did not exceed 2 µg.

5) recovery of DNA from the sucrose gradient fractions was between 75% (DABA) and 95% (EtBr).

The amount of DNA per cell deserves some comment. The cells contain more than twice the amount of DNA found in diploid Drosophila cells. However, at least cytologically, the cell line from which the Kc cells used in our laboratories in Tübingen are derived was initially diploid (ECHALIER and OHANESSIAN 1969).

It is obvious that at least some part of the genome must have been amplified. However, there is a number of mechanisms by which amplification could occur. Genome duplication, aneuploidy and selective amplification of particular sequences have all been described for Drosophila cell lines (KAKPAKOV et al., 1969, FACCIO DOLFINI 1976, HALFER 1978; POTTER et al., 1979, HALFER et al., 1980; for review see SANG 1981). In addition there is no guarantee that the cell line which is cultured in our laboratories in Tübingen is homogeneous. The line is not of clonal origin, and has been in continuous culture for 12 years (SANG 1981), and could conceivably consist of an equilibrium mixture of more than one cell type (see e.g. KAKPAKOV and POLVKAROVA 1977). It is possible to conceive many possible schemes, including combinations of the above mechanisms, which could give rise to the observed DNA content.

However, one of the simplest explanations for the observed DNA content would be if it derived from a logarithmically growing population of tetraploid cells, with about 30% of the cells in the G2 phase of the cell cycle. The

mean DNA content of nuclei in such a population would be approximately the observed amount (0.91 pg per nucleus). The higher DNA content in cells compared to nuclei might be explained by presuming that some cells are multinucleate. In addition, mitotic cells and mitochondria are lost during the isolation procedure. However, without further cytological or sequence organisation data there is no way of deciding whether such a simple description of the observed data bears any resemblance to the authentic state of genome organisation within these cells.

R.9.1.6 RNA Content of Drosophila Kc Cells

The ethidium bromide (EtBr) method described in section M.1.3 proved adequate for the task of estimating the amount of RNA in Kc cell fractions, but only after solving two major problems in interpreting the data.

Problem 1: finding the appropriate fluorescence blank for the different assay treatments.

The method requires measuring the fluorescence enhancement of ethidium from RNA containing samples which have been subjected to different combinations of degradative enzymes (DNAse, RNase, Protease, see M.1.3 p. 22). In order to be able to estimate the fluorescence enhancement caused by an EtBr-nucleic acid interaction, it is necessary to know the intrinsic fluorescence intensity of EtBr in the medium (buffer or buffer + enzyme) in which the nucleic acid is dissolved or suspended (fluorescence blank). The interval on the ordinate labelled intrinsic fluorescence in the RNA calibration regression shown in fig. 46 shows the magnitude of this quantity in the absence of any added enzymes.

If this blank value were the same for all treatments of the RNA it would not be necessary to measure it to estimate

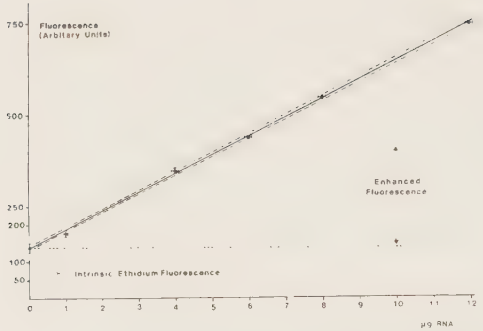


Fig. 46 **Calibration curve for the determination of RNA using the EtBr method.**
The absolute fluorescence and fluorescence enhancement obtained in buffer, in the absence of any degradative enzymes, using the amounts of RNA indicated on the abscissa is shown. The solid line is the regression fitted by the method of least squares. The dashed lines indicate the 95% confidence limits for the regression. The data points are the means of duplicate or multiple values. The vertical error bars show the range (duplicate values) or 90% confidence limits (multiple determinations). The horizontal error bars indicate the errors which would occur with a pipetting error of ±0.5 µl below 10 µl and of ± 1.0 µl in the range 10-100 µl.

the amount of RNA, since it is removed by calculation (see section M.1.3, p.22). If it were to vary, then the fluorescence enhancement in each different treatment would have to be calculated by subtraction of the corresponding blank, and these would have to be measured separately. Unfortunately as table 45 shows, the direct measurement of the fluorescence intensity of the various fluorescence blanks was of insufficient precision to allow a decision as to whether there really was a difference between them.

The higher of the two PBS measurements (upper end of range in table 45) has a value very similar to that of the enzyme containing blanks. If the lower of the two measurements were in error, then there would be very little difference between the blank value of the PBS and that of the PBS+enzyme

blank and individual correction would not be necessary. If either the lower of the two values or the mean value of the PBS blank were to provide a more correct estimate, then individual correction would obviously be required. This situation would probably have been avoided if there had been more adequate replication of the blank values.

Treatment	Mean Fluorescence (Arbitrary Units)		Range	Nr. Determinations
PBS (stood 30 min)	136.7		124.8 - 148.6	2
PBS (stood 3 hr.)	133.1		119.0 - 147.2	2
Mean PBS	134.9			
PBS + Proteinase K	144.1	}		
PBS + RNase	149.1	}	SINGLE VALUES ONLY	
PBS + RNase + Proteinase K	149.0	}		
Mean Value Blanks all treatments (even weighting of treatments)	142.4	S.E. 7.3		

Table 45 **Fluorescence intensity of fluorescence blanks**
The exact composition of each blank is given in table 4 in Methods and Materials, p. 22.

The large difference between the two PBS treatments (approx. 10%), was quite unrepresentative of the reproducibility usually achieved with this method: in another series of measurements in which 10 replicate samples were measured, the coefficient of variation was less than 1%.

A solution to this problem was found by examining the consistency of the estimates of the amount of fluorescence attributable to RNA in the sucrose gradient fractions of the

RNP extract. These fractions were chosen which did not appear to contain any endogenous RNase activity (see this section part b). A measure of consistency was obtained by comparison of the results obtained with each of the the three different methods of estimation described in section M.1.3.

i.e the differences in fluorescence intensity between

1) a sample kept at room temperature (R.T.) without RNAase and a duplicate sample incubated at 37°C with RNAase

2) a sample incubated at 37°C without RNAase and a duplicate sample incubated at 37°C with RNAase

3) a sample incubated at 37°C with Proteinase K, but without RNAase and a duplicate sample incubated at 37°C with both enzymes.

were compared. The measurements of fluorescence intensity were all made at room temperature.

Since method 3 (M.1.3. p.22) involves the use of a different combination of fluorescence blanks from methods 1 and 2, one would expect an inappropriate choice of blank value to lead to discrepant results, whereas the correct blank value should give results which are in agreement with one another. The results of this examination are shown in table 46.

It is clear from table 46 that the variation in the estimates of RNA-attributable EtBr-fluorescence using individual fluorescence blank corrections is smaller by a factor of 3 than that found assuming that all treatments have the same blank value. All further estimates in this section have therefore been obtained by using the individual treatment fluorescence blanks.

A) Individual Fluorescence Blank Correction				B) Same Fluorescence Blank for all treatments		
Fraction Nr.	Mean Fl. enhance (arbitrary) (units)	S.E.	Coeff. of Variation ((S.E./Mean)x100	Mean Fl. enhance (arbitrary) (units)	S.E.	Coeff. of Variation ((S.E./Mean)x100)
9	45.0	8.3	18.4	33.6	13.7	41.0
8	40.8	1.6	3.8	29.6	3.8	12.8
7	37.1	3.8	10.2	25.9	1.7	6.6
6	40.4	2.0	4.9	29.3	6.8	23.2
5	38.4	2.8	7.3	27.3	11.0	40.1
4	32.4	1.4	4.2	21.3	6.7	31.2
3	22.4	1.7	7.6	11.7	2.3	19.8
Mean Coefficient of variation			8.6			24.9

Table 46 **Effect of different fluorescence blank corrections on the agreement between estimates of ethidium Bromide(EtBr) fluorescence attributable to RNA-EtBr interaction.**
The RNA was contained in hnRNP isolated by sedimentation velocity fractionation in sucrose gradients similar to the one shown e.g. in RISAU et al., 1983, fig. 2, except that 9 1.2 ml fractions rather than 18 0.6 ml fractions were collected. Fraction 3 was from the top of the gradient and fraction 9 from the bottom. Fractions 1 and 2 were omitted from the analysis because the fluorescence enhancement to which they gave rise was negligible. They may have contained RNA, but if this was very extensively degraded it would not cause fluorescence enhancement.

Problem 2: endogenous nucleases.

Apart from showing that individual fluorescence blanks must be measured, the results shown in table 46 also show how reliable this method can be. 5 pipetting steps were involved in making up each sample treatment. Aliquots of each sucrose gradient fraction were subjected to six different kinds of treatment. The fluorescence determinations on these treatments were then used to calculate the three independent estimates of RNA-attributable fluorescence whose mean is shown in table 46 panel A. Taking the fluorescence blank into account, each of

the three estimates is arrived at by computing the difference between two differences. When one considers that the sources of error in making up individual treatments and in computing differences are cumulative then obtaining a mean coefficient of variation between the three estimates of under 10% attests the robustness of the method.

This level of agreement was not obtained using RNA containing cellular fractions other than those purified by sucrose gradient centrifugation. There is a trivial explanation for this lack of agreement and that is that there was appreciable endogenous nuclease activity in the cruder extracts. This is clearly illustrated in table 47 below. Rows 1 and 2 of this table are the controls, and show that there was no detectable RNAase activity in any of the buffers used (compare second and third columns of figures from the left with first column, column 1), although the samples contained RNAase sensitive material (column 4). Row 3 shows that after 3 hours at room temperature 10% of the nucleic acid attributable fluorescence is lost from the hnRNP fraction and that incubation at 37°C for 30 min results in somewhat greater loss: 16% or 9% for the hnRNP and nuclear fractions respectively. However it is clear from the last column of rows 3 and 4 that in both cases the extent of the loss is of at most 20% of the total amount of RNase sensitive material present.

RISAU (1983) has also produced evidence for endogenous nuclease activity in hnRNP extracts (cf. his fig. 13), His observations showed that long RNA molecules were converted to shorter ones, resulting in altered sedimentation properties of hnRNP particles, but gave no indication of the nature final RNA product size. The decline in fluorescence enhancement suggests that very extensive degradation of (some) RNA molecules is likely to be taking place. RNA molecules as short as 50 bases give rise to fluorescence enhancement (KARSTEN and WOLLENBERGER 1972), so it is possible that degradation to single nucleotides may be taking place.

Source of sample	Fluorescence intensity attributable to ethidium-nucleic acid interaction after			
	standing R.T. 30 min	standing R.T. 180 min	incubation 37°C 30 min	incubation 37°C 30 min + RNAase
	(in arbitrary units)			
5S RNA Standard (4 µg)	203.5	210.8	201.4	-0.6
Representative RNP fraction from sucrose gradient	58.1	59.1	56.1	17.9
hnRNP Extract	268.1	241.1	216.1	60.4
Nuclei	1784	n.m	1622	993

(n.m.=not measured)

Table 47 **Evidence for endogenous nuclease activity in nuclei and nuclear extracts of Kc cells.**
The composition of the individual treatments is described in section M.1.3.

Since the enhanced fluorescence of ethidium derives from binding to both DNA and RNA it seems reasonable to suspect that both DNAase and RNAase might be responsible for the decreases in fluorescence seen in the crudest fractions. As already remarked in section R.9.1.5 the EtBr estimates for DNA in cells and nuclei were lower than those found using the DABA method, and simple calculation shows that discrepancy could easily be accounted for by the extent of nuclease action seen during incubation at 37°C. The EtBr estimates of the amount of DNA in the RNP extract and in the sucrose gradient pellet were slightly higher than those estimated using the DABA method, so the simplest interpretation of this data is that the decline of fluorescence in these samples was solely attributable to RNAase.

In the light of these considerations it was possible to find empirical correction factors for the data from 37°C

incubations made in the absence of Proteinase K which result in agreement between the DABA data for DNA and between estimates 1 and 2 of the amount of RNA derived EtBr fluorescence enhancement. They are listed in table 48. The estimates of RNA made in this section are based on this interpretation of the data.

When the crude fractions were treated with Proteinase K, the estimates of RNA were much lower, by about 30% (method of estimation 3 - see p.388) than those obtained in its absence. There was no evidence of RNAase activity in the Proteinase K (cf. the good agreement between the three methods of estimation using the sucrose gradient purified hnRNPs), and since the DNA estimates provided by the EtBr and DABA methods were substantially in agreement, most of the discrepancy must be attributable to RNAase. A possible explanation is that Proteinase K is destroying proteins which protect RNA from nuclease attack before eliminating endogenous nuclease activity, thereby exposing protected regions of RNA to nucleolytic attack. Method of estimation number 3 could therefore not be used with these crude fractions.

Fraction	Proportion fluorescence **decline** attributable to endogenous	
	DNAase	RNAase
Cells	38%	62%
Nuclei	67%	34%
hnRNP Extract	-	100%
hnRNP Extract Gradient Pellet	-	100%

Table 48 **Estimates of the effect of endogenous nuclease on the decline of fluorescence intensity attributable to EtBr-nucleic acid interaction following incubation of sample at 37°C in the absence of added nuclease.**

The presence of nuclease activity in these crude fractions is not particularly suprising, since no special

precautions were taken to circumvent it. On the other hand, the authors of the original paper from which this method was taken did not take any special precautions either (KARSTEN and WOLLENBERGER 1972). Perhaps in the tissues these authors examined the level of nuclease activity was very much less than in Kc cells.

* *

Once these difficulties in interpreting the data had been overcome, the amount of RNA in the various cell fractions could be estimated very precisely, because the calibration data was excellent, as shown in fig. 46. The problems of bad fit encountered with low amounts of DNA using the ethidium method were not encountered with RNA. This is not a contradiction, since the calibration curves are based on completely separate sets of data.

Table 49 documents the quantities of RNA that were found in each of the cell fractions.

Five major conclusions follow from the data shown in table 49.

1) The RNA content of the nuclei from Drosophila Kc cells is equal to or slightly exceeds the DNA content.

2) At the very least 30% of the nuclear RNA is extractable into a fraction (hnRNP fraction) which has been shown by RISAU (1983) to contain 70% of the rapidly labelled nuclear RNA.

3) At least 20% of the RNA in the hnRNP fraction (5% of the total nuclear, 1% of the total cell RNA) can be recovered in the sucrose gradient fractions containing hnRNP (material with sedimentation coefficients between 30S and 160S).

Fraction	Amount RNA measured	Amount RNA in original fraction.	RNA per cell or cell equivalent.
	(µg)	(µg/ml)	(pg)
Cells	22.0 (21.6 - 22.4)	439.6 (431.4 - 448.0)	6.06 (5.75 - 6.40)
Nuclei	13.6 (13.4 - 13.8)	680.5 (669 - 692)	0.89 (0.81 - 0.97)
RNP Extract	4.08 (4.00 - 4.16)	204 (200 - 208)	0.27 (0.24 - 0.29)
Nuclear Residue	By difference	480	0.62
RNP Extract Sucrose gradient pellet	3.01 (2.92 - 3.09)	60.2 (58.4 - 61.8)	0.08 (0.07 - 0.09)
Sum of all fractions from Sucrose Gradients (=recovery non-pelletable RNA from RNP)	6.84 (4.60 - 8.05)	n.a (3.2 - 8.8)[1]	0.09 (0.08 - 0.10)

Notes [1] n.a. not applicable: the range of RNA concentrations found in the gradient is given.

Table 49 **Estimates of the amount of RNA in Drosophila Kc cells and Kc cell fractions.**

Data is from a single determination, run in parallel with DNA determination Nr. 3 (see Table 43, p.378). In columns 2 and 3 95% confidence limits for the mean value of the estimate are shown in brackets (see M.7.3). In the last column, the limits are worst case estimates using the limits shown in columns 2 and 3 and the 90% limits of the particle counts shown in Table 39.

4) Only 60% of the applied RNA is recoverable from the sucrose gradients.

5) The weight ratio of RNA to DNA in material recovered from the sucrose gradients is greater than 10 to 1, suggesting that contamination of hnRNP by chromatin is negligible.

The only one of these conclusions that requires any comment here is the low recovery of RNA from the sucrose gradient fractions. Approximately 40% of the RNA applied to the sucrose gradients could not be recovered after centrifugation. For DNA the loss was negligible (see fig. 45). Three possible explanations, none of which are mutually exclusive, spring to mind:

1) Extensive RNAase degradation before or during centrifugation. As shown in part b) of this section the hnRNP fraction does contain endogenous RNAase activity, so this is a feasible explanation. However, the high molecular weight of the RNA which is found in the hnRNP fraction (RISAU et al. 1983) argues against this possibility, unless some RNA molecules are degraded processively while others are left intact.

2) Absorption of hnRNP material to the walls of the centrifuge tube during centrifugation. This a well known problem to molecular biologists who employ centrifugation methods. However to be able to explain the present results, each tube would have to be able to absorb about 70 µg of RNA and probably a four fold greater amount of protein (see R.9.2.1). Assuming that each tube has a surface area of approx 36 cm^2 (diam.=0.75 cm x π x length 12cm) then this would mean a capacity of 8 $\mu g/cm^2$. A capacity of about 1 $\mu g/cm^2$ has been observed for PVC (see section R.7.4.3). Nitrocellulose may have a greater protein binding capacity (R. HALLMAN pers. comm), so that a significant amount, but probably not all, of the loss might be attributable to this mechanism.

3) A trivial explanation for some of the non-recovery might be the inability to scrape all of the highly compact pelleted material from the bottom of the centrifuge tube. This effect however cannot explain the differential recovery of RNA and DNA containing material and so could only explain away part of the observed loss.

In the rest of this chapter the significance of the results in section R.9.1 in interpreting antigen frequency is described. A summary of the absolute and relative nucleic acid content of Drosophila Kc cells is given in table 50 in the following section (R.9.2.1).

R.9.2 Implications of macromolecular composition of Kc cell nuclei

R.9.2.1 Non-immunologically determined upper limits of hnRNP associated antigen frequency in Kc cell nuclei

The data from section R.9.1 about the composition of the hnRNP fraction (see table 50) allows some approximate predictions to be made about the gross frequency range in which various classes of protein might be expected to occur in the Kc cell nucleus.

The proportion of total nuclear protein or RNA found in the hnRNP fraction is 13% or 30% respectively. However, only about 70% of the total rapidly labelled RNA was extractable into this fraction (RISAU et al., 1983). Correcting for this, and assuming that RNA and RNA associated protein are extracted equally efficiently, gives figures of 20% and 40% for the proportion of total nuclear protein and RNA which form part of hnRNP if these could be extracted with 100% efficiency. The amount of DNA, presumably in the form of chromatin, which contaminates the hnRNP fraction is small (3% of the total DNA in the nucleus). Even assuming a generous 2:1 protein to DNA ratio in chromatin, DNA associated protein could make up at

most 5% of the protein in the hnRNP extract. Thus the proportion of total nuclear protein which might potentially be associated with hnRNA, as judged by the criterion of co-extractibility is at least 19%.

Fraction	DNA Amount per Cell (pg)	RNA	RNA/DNA	% Nuclear RNA
Cells	1.03	6.06	5.9	-
Nuclei	0.91	0.89	1.0	100
hnRNP Extract	0.033	0.27	8.2	30
Gradient Pellet	0.023	0.08	3.5	9} gradient
Gradient hnRNP	0.007	0.09	13.0	10} recovery

Table 50 **Absolute amounts of DNA and RNA, ratio of RNA to DNA and proportion of RNA in Drosophila Kc cells and cell fractions**

In an experiment using a separately prepared but very similar extract to that used for the analysis shown in section R.9.1, analysis of the protein content of fractions obtained by sedimentation velocity separation in sucrose gradients revealed that 65% of the recovered protein sedimented in fractions containing particles of size > 40S, fractions containing more than 90% of the rapidly-labelled RNA. The low recovery of RNA (and presumably protein) from the gradients (60%, see R.9.1.6) make it uncertain whether this figure can be extrapolated to the behaviour of all the protein in the extract, but I will assume that this is legitimate. It follows from this assumption that the proportion of total nuclear protein physically associated with hnRNA ("hnRNP protein")is 19% x 0.65 = 12.5% (= 1.45 pg/nucleus). The amount of nuclear RNA in hnRNP was estimated above to 40% of the total (=0.36 pg/nucleus). This gives a protein:RNA ratio for Drosophila hnRNP of almost exactly 4:1, which is very similar to that

reported for the hnRNPs of other organisms (MARTIN et al., 1973).

If I assume that at least 80% of the mass of hnRNP protein can be attributed to the major packaging proteins (see e.g. fig. 5 lanes 10/11 in RISAU et al., 1983) then this would mean that at most 2% of the total protein in the Kc nucleus can be made up of non-packaging hnRNP protein. Inspection of figure 5 of RISAU et al., (1983) reveals at least 20 minor polypeptides cosedimenting with hnRNP in addition to the major putative packaging proteins. This suggests that individual species of non-packaging proteins will, on average, make up about 0.1% of the total nuclear protein. For such a protein with a M.W. 50,000 dalton the number of moles/nucleus may be calculated as follows:

$$\text{moles/nucleus} = \frac{\text{mass of total nuclear protein (g)} \times \text{proportion of protein}}{\text{Molecular weight of protein}}$$

$$= \frac{1.17\ 10^{-11} \times 10^{-3}}{5\ 10^{4}} = \frac{1.17\ 10^{-14}}{5\ 10^{4}}$$

$$\approx 2\ 10^{-19} \text{ moles}$$

from which, by multiplication with Avagadro's number the frequency in molecules/nucleus can be obtained

$$\text{molecules/nucleus} = 2\ 10^{-19} \times 6\ 10^{23}$$

$$\approx 10^{5} \text{ copies}$$

The frequency of the major packaging proteins may be estimated by assuming that the most frequent polypeptide component has a molecular weight of $5\ 10^{4}$ daltons (RISAU et al., 1983) and makes up no more than 33% by mass of each particle (cf. e.g. RISAU et al., 1983). On the basis of the figures above this implies

$$4.0\ 10^{6} \text{ copies/nucleus}$$

of this component. Assuming some fixed stoichiometry in the particles, the less frequent packaging components would be expected to occur as submultiples of this frequency, a polypeptide occurring once per particle would probably have a frequency of about

10^6 copies/nucleus

(see BEYER et al., 1977). For comparison, histone molecules are expected to have have a frequency of approximately

10^7 copies/nucleus.

These estimates are summarised in table 51 below.

It must be stressed that these estimates are very approximate. However, it is very unlikely that any of the estimates are in error by as much as an order of magnitude. Their validity rests mainly on the accuracy of the nucleic acid estimates and the expectation that protein:nucleic acid ratios for Drosophila resemble those of other species. They also assume that the non-packaging hnRNP proteins are more or less quantitatively extracted into the hnRNP fraction. RISAU (1983) has demonstrated (see his fig. 7) that for at least 2 of the non-packaging proteins (those identified by the X4 and P11 antibodies) this condition is satisfied. Were they to be in some sort equilibrium with or sequestered within other nuclear structures (see e.g. DANGLI et al., 1983), as may be the case for the S5 or T7 antigen (RISAU 1983), the frequencies listed in table 51 would be in error in terms of absolute number per nucleus. They would however continue to give correct estimates of the amount of these proteins physically associated with hnRNA.

The ratio of packaging to non-packaging proteins I have chosen is somewhat arbitrary and the amount of individual non-

packaging species clearly varies. It would be desirable to have data from densitometric analyses to provide more quantitative evidence for the assumed ratios. However, I would judge it unlikely that any individual species occurred with a frequency as high as 1/4 that of the least frequent putative packaging protein, which would set an upper limit of 3 10^5 copies/nucleus for the most frequent non-packaging protein. Thus the estimates in this section tend to the upper limit of protein frequency.

Nuclear protein species	Amount (pg/nucleus)	% total nuclear protein	molecules/ nucleus
Total nuclear protein	11.7	100	
DNA associated protein	1.8*	15	
Histones (aggregate)	0.9*	8	
Individual species (H2A etc.,)	0.2*	1.6	10^7
non-histone (aggregate)	0.9	8	
Protein in hnRNP (aggregate)	1.4	12.5	
Packaging proteins (aggregate)	1.2	10.5	
Packaging proteins (individual -monomer- species)	0.1 - 0.4	1 - 3.5	10^6 - 4 10^6
Non-packaging proteins (aggregate)	0.2	2	
Non-packaging proteins (individual species)	0.01	0.1	10^5
Other nuclear protein (includes nucleolar protein)	8.5	72	

* Assumed by analogy with other organisms

Table 51 **Estimated frequency of protein associated with nucleic acid in Drosophila Kc cell nuclei**
See text for details.

Given 0.36 pg hnRNA/nucleus and a median hnRNA size of 3000 bases implies a nascent and immature transcript frequency on the order of 10^5/nucleus. The kinetic complexity of Drosophila hnRNA has been estimated at about 2 10^4 (LEVY-W. et al., 1976), presumably many of which occur at very low frequency. However even if the complexity was represented by the equivalent of only two full-length transcripts per nucleus, this would account for 40% of the copies. It would be neccessary to have more detailed information (not yet available) about transcription rates, hnRNA frequency classes and the ratio of nascent:immature RNA molecules to be able to provide a realistic description of nuclear RNA metabolism.

However, if the hnRNA molecule number estimate is correct, it suggests that non-packaging proteins would occur with a average frequency of about 1 molecule non-packaging protein/nascent or immature transcript. However, cytological evidence suggests that some of the immunologically identified non-packaging proteins are not uniformly associated with transcripts (see R.10, KABISH and BAUTZ 1983, DANGLI et al., 1983), so that several copies might present on some transcripts and none on others.

In contrast to the non-packaging proteins, assuming that the most frequent packaging protein occurs with a copy number of 4 per elementary hnRNP particle (BEYER et al., 1977), about 40 copies of each packaging polypeptide would be expected to occur on a 3000 base transcript, at an average interval of 1 particle per 75 bases.

The importance of the above estimates is that they have been made without recourse to any immunological data. Thus they provide independent estimates (albeit very crude ones) for comparison, with the results of the immunological estimates presented in the next section R.9.2.2.

R.9.2.1.1 Comparison with other organisms

It is perhaps worth pointing out that the macromolecular composition of Drosophila Kc cell nuclei appears to be different from the only two vertebrate species for which I have been able to find comparable data. As table 52 shows, in terms of their high protein:DNA ratio, the composition of Drosophila Kc cell nuclei more closely resembles that of yeast than that of rat or mouse.

Source of nuclei	genome size (base pairs)	DNA : RNA: Protein (relative amounts)			Reference
Saccharomyces cerevisiae	$2\ 10^7$	1	2.5	16	BHARGARVA & HALVORSON (1971)
Drosophila melanogaster	$(3\ 10^8)$ * $6\ 10^8$ **	1	1	13	This work
Rat Liver	$5\ 10^9$	1	0.2	4.4	TATA (1974)
Mouse (L cell)	"	1	0.5	?	BRANDHORST & McCONKEY (1974)

* Normal organism **Kc cell

Table 52 **Comparative macromolecular composition of nuclei from species with different genome sizes.**

The higher RNA content can be explained if it is assumed that the proportion of both yeast and Drosophila genomes which is transcriptionally active is greater than that of rat liver cells. The higher protein content of the nuclei of the two invertebrate species can be explained, at least in part, by the higher protein:nucleic acid ratio found in hnRNP as opposed to chromatin (defined as DNA and DNA associated protein extractable from the nucleus). Simple calculation shows that in invertebrate species the frequency of RNA associated proteins is likely to be at least as great as that of DNA associated non-histone proteins, In contrast, in vertebrates, if the data in table 52 is representative, DNA

associated non-histone protein will be more abundant by a factor of 2 to 5 than RNA associated non-packaging protein. The high content of hnRNA associated protein relative to other nuclear components might be a contributing factor to the high relative frequency with which antibodies against them (probably most "class I" antibodies in the bank of SAUMWEBER et al., 1980) have been obtained. Perhaps if vertebrate nuclei were used as a source of antigen the relative yield of antibodies against DNA associated antigens would be greater.

R.9.2.2 Immunologically determined lower limits and "most likely" estimates of hnRNP associated antigen frequency in Drosophila Kc cell nuclei

The signal obtained from IRMA (or ELISA), calibrated to allow the number of antigen molecules adsorbed to a solid phase to be measured, provides only a logical "lower limit" to the amount of that antigen which is contained in the fraction used to coat the well. This is because the adsorption efficiency of an antigen is unknown and, as I showed in sections R.7.4.1, R.7.4.2 and R.8.2 may vary from antigen to antigen in an unpredictable manner. The results obtained in those sections were restricted either to the highly abundant histone 1, or to artificial mixtures containing IgG, and all 3 studies used polyclonal antibody. However, there is also evidence for variable absorbtion efficiencies of monoclonal antibody detected Kc cell nuclear antigens from sonicated Kc cell nuclei. For 6 antigens, the absolute amount of antigen adsorbed from this source has been used to calculate the number of antigen molecules per Kc cell nucleus assuming adsorption efficiencies of 50%, 5% or 0.5%. Table 53 shows the results of these calculations together with estimates of the frequency of 3 of the antigens made by RISAU (1983), using an independent method.

	ANTIGEN					
	S5	T7	P46	P11	P12	X4
Antigen Molecules adsorbed to well surface x 10^{-9}	1	2	0.5	6.7	0.1	~1.5
	NUMBER OF ANTIGEN MOLECULES PER Kc CELL NUCLEUS ESTIMATED AT VARIOUS ANTIGEN ADSORPTION EFFICIENCIES					
Antigen adsorption efficiency						
50%	1000	2000	500	6700	100	~1500
5%	10^4	$2\ 10^4$	5000	$6.7\ 10^4$	1000	$\sim 1.5\ 10^4$
0.5%	10^5	$2\ 10^5$	$5\ 10^4$	$6.7\ 10^5$	10^4	$\sim 1.5\ 10^5$
RISAU (1983) estimate	$2.9\ 10^5$–$1.4\ 10^6$	n.m.	n.m.	$3.7\ 10^4$–$1.8\ 10^5$	n.m.	$5.6\ 10^4$–$2.8\ 10^5$

n.m. not measured

Table 53 **Immunological estimates of antigen frequencies in Drosophila Kc cell nuclei using solid phase assay (IRMA)**
The number of antigen molecules adsorbed to the well surface is taken from table 34, p.340. 20 µg sonicated Drosophila Kc nuclear protein (Biuret) = 10 µg (Bradford, data not shown), equivalent to approximately $2\ 10^6$ nuclei was incubated in each well and gave rise to the number of immunologically detectable adsorbed molecules indicated in the topmost row of figures. The value for X4 is taken from the X4:S5 IRMA signal ratio found in a number of assays under similar conditions. It has not been accurately estimated from the detector signal extrapolated to infinite detector concentration as have the others. The values in the lower part of the table indicate the numbers of antigen molecules/nucleus in original nuclear preparation assuming the adsorption efficiencies indicated in the leftmost column of the table. RISAU (1983) estimates indicate range of frequencies estimated from amidoschwarz staining intensity of spot corresponding to antigen in a 2D gel.

The adsorption efficiencies for which the calculations whose results are shown in table 53 have been made were not chosen arbitrarily. The 50% adsorption efficiency figure represents a rather unrealistic upper limit to the amount of protein that could adsorb to the well surface during the

antigen-coating incubation. It is the limit set assuming a diffusive transport mechanism, a diffusion coefficient of approx 10^{-6} cm^2 s^{-1} and neglecting all other sources of interference. The 5% adsorption efficiency figure represents the observed adsorption efficiency of bulk nuclear protein (see R.7.4.3). The 0.5% adsorption efficiency figure is included to show that for the S5 and X4 antigens an efficiency of this order must be assumed to bring estimates based on the IRMA signal into the frequency range found by RISAU (1983) using an independent method of measurement (see above and table legend).

The estimates obtained by RISAU (1983) for the P11 and X4 antigens were very clear cut. The polypeptides corresponding to these antigens electrophoresed to areas of the 2D-gel gel in which they were well separated from all other polypeptides, thus giving rise to single isolated spots upon staining. This allowed their identification and quantitation of the amount of dye staining (fig. 19 of RISAU 1983). However, to bring the solid phase assay results for each antigen into agreement with the RISAU estimates it is necessary to assume different absorbtion efficiencies. Inspection of table 52 shows that assumption of an adsorption efficiency in the range 2% - 10% is required to obtain agreement between the two sets of results for the P11 antigen. In contrast, for the X4 antigen an adsorption efficiency in the range of 0.3% - 1.4% would have to be assumed. This suggests a difference in adsorption efficiency of about a factor 5 between these two antigens, although it might be as little as 2 and as great as 30. At any rate, the adsorption efficiency of the X4 antigen appears to fall significantly below that of bulk protein.

If RISAU (1983)'s estimate of the S5 antigen's frequency is accepted without reservation, the efficiency with which the S5 antigen adsorbs to solid phase must be still lower than that of the other 2 antigens, on the order of 0.1%. This may be the correct explanation but, in the case of this antigen,

it is also possible that the RISAU (1983) estimate might be in error (overestimate) as the result of spot misidentification, since the S5 antigen electrophoreses in a region congested by a number of other spots. A further indication that the RISAU estimate may be an overestimate comes from one dimensional gel SDS polyacrylamide gel analysis which shows that only small amounts of this antigen (relative to the putative packaging proteins) can possibly be present in hnRNP extracts (there are no abundant polypeptides in the S5 M.W. region). On the other hand it is possible that this antigen is only inefficiently extracted into the hnRNP fraction (cf. RISAU 1983 fig. 7). In view of these ambiguities the question of the frequency of the S5 antigen in Kc cell nuclei and of the efficiency of its adsorption to PVC solid phase from nuclear sonicates must remain open.

It is comforting that the RISAU (1983) estimates suggest frequencies for these antigens on the order of 10^5 per nucleus, because it indicates that the assumptions made about the macromolecular composition of the nucleus in the previous section are not grossly in error. Further support for a frequency in this range is provided by the magnitude of the assay signals obtained from a number of the hnRNP associated antigens when sedimentation velocity fractionated hnRNP fractions are used as a source of antigen. The antigen frequency/nucleus which can be calculated on the basis of these signals is summarised in table 54.

Because of the large number of uncertain assumptions involved in making the calculation upon which the figures in table 54 are based, the estimates presented there can only be taken as order of magnitude approximations. However, it seems reasonably certain that the logical minimum estimate (50% adsorption efficiency) of antigen molecules/nucleus using the material in these fractions as a source of antigen is greater than that obtained using whole nuclei as a source of antigen.

	ANTIGEN						
	X4	S5	P11	T7	P46	Q18	V4
at adsorption efficiency	Antigen molecules/Kc nucleus						
50%	$3.2\ 10^4$	$2.4\ 10^4$	$4.0\ 10^4$	10^4	6000	2000	800
15%	$1.0\ 10^5$	$0.7\ 10^5$	$1.3\ 10^5$	$3.3\ 10^4$	$2\ 10^4$	7000	2600
RISAU (1983)	$5.6\ 10^4$ - $1.4\ 10^5$	$2.9\ 10^5$ - $1.4\ 10^6$	$3.7\ 10^4$ - $1.8\ 10^5$	- - - not measured - - -			

Table 54 **Estimates of amounts of hnRNP associated antigens in Drosophila Kc cell nuclei estimated from amounts in particles fractionated by sedimentation velocity**

The data on which these estimates are based is taken from fig. 2 of RISAU et al., (1983). Estimates of the number of molecules adsorbed to solid phase if the total content of each of fractions from the centrifuge tube were assayed (M) were calculated according to formula:

$$M = S \times A \times D \times R \times St \times Ex$$

where:

- M = total number of antigen molecules adsorbed under conditions of assay.
- S = Sum of assay signals from all assayed fractions
- A = 1/specific activity of detector = 10^5 molecules/cpm (assuming a specific activity of 20 mCi/mg)
- D = dilution correction (180 for P11, 60 for other antigens: 0.6 fractions diluted 1/30 (P11) or 1/10 (the others) and assayed in 100 µl aliquots)
- R = recovery correction (4 for S5 and X4, assumes 50% recovery and 50% in gradient in gradient pellet, 2 for others assumes 50% recovery)
- E = correction for inefficiency of detector binding (5, see R.7.4.5)
- St = correction for stoichiometry of detector binding to monoclonal antibody (=0.25 for P11 and Q18 - IgG2a - or 0.5 for others)
- Ex = correction for inefficient extraction of hnRNP extract from nuclei (1/0.7 = 1.4)

Division of M by 10^9 (the number of nuclei applied to each tube for fractionation) gives the number per nucleus.

Assuming the same absorbtion efficiency the minimum estimates for the frequency/nucleus are between a factor 7 (P11), or 25 (X4 or S5), greater than from crude nuclei (compare top line in fig. 54 with top line in the lower half of fig. 53). It seems unlikely that discrepancies of this magnitude can be entirely attributed to errors in the assumptions used for calculation. I would like to suggest that it is more probable that the discrepancy results from real differences in antigen adsorption effiency from the two different sorts of extract.

This suggestion is based on the consideration that particles with an S value of approximately 100S, a molecular weight of 5 10^6 (3 kbases RNA ≈ 10^6 dalton plus ~ 4 10^6 dalton protein, see previous section) and a partial specific volume of 0.65 would be expected to have a diffusion coefficient of approximately 10^{-7} cm^2 s^{-1} (see equation in M.7.5 at top of p. 88). Under the conditions of antigen adsorption used for the assay (1 hour at 0°C), such particles would be expected to adsorb with an efficiency of about 15%. The middle line of table 54 shows that, when such an efficiency is assumed, the antigen/cell estimate obtained from the adsorption data for the X4 and P11 antigens gives reasonable agreement with that obtained by RISAU (1983) (bottom line of table 54). The S5 titre is again considerably lower than that of RISAU but this, as I argued earlier, may be attributable either to an erroneous RISAU estimate or to poor extraction of this antigen into the hnRNP extract.

The rate of transfer of hnRNP particles to the solid phase bound antibody X4 in the "reverse RIA" method described in RISAU et al., 1983, appears to have been about 7% in one hour (see inset in fig. 4b in that article), which is about half the rate supposed above. However, it is possible to imagine a number of explanations which might explain this apparent discrepancy. For instance, not all the hnRNP might contain the antigen. If only one in every two hnRNP were to contain the X4 antigen, which is certainly conceivable, this would suffice to give agreement between the two sets of

results. The specific activity of the RNA in these experiments was low, so that another possibility is that particle binding to adsorbed antibody may have been becoming saturated. At any rate, the rate of transfer observed in reverse RIA does not contradict the notion that transport to the vessel walls is diffusion controlled.

If my interpretation of the results in table 54 bears any relation to reality, then it would mean that the absorption efficiency from these fractions would be a factor 2-10 higher for the P11 antigen and a factor 10-50 fold higher for the X4 antigen. It does not seem unreasonable to expect such increases. The particles obtained by sedimentation velocity fractionation should be highly enriched, if not homogeneous. In consequence less competition for binding sites on the solid phase between these particles and other molecules would be expected. This in turn would imply that the the X4 antigen (or more probably an X4 containing particle) is selectively excluded from adsorption, relative to the P11 antigen, when crude nuclei are used as antigen source.

Since it is possible to suggest a physical mechanism for the absorbtion efficiency which would bring the solid phase assay results using the fractionated hnRNP particles into agreement with RISAU's, I regard this as a "most likely" estimate. Furthermore it should be possible to test whether the predicted adsorbtion efficiency agrees with that observed. Once the maximum plate capacity for these particles has been determined it should be simple to measure the adsorption rate, and hence efficiency, using the method outlined in part R.7.3.3. Indeed, since the sedimentation coefficient of the particles is known, by using solid phase assay to measure the diffusion constant of the particles it should in principle be possible to measure their molecular weight.

A converse effect of accepting my interpretation of the results presented in table 54 is that particles sedimenting at different rates would be expected to have different diffusion

coefficients and hence adsorption efficiencies. No correction, or a uniform correction, such as the 15% I have used for the purposes of illustration in table 54, would be expected to cause overestimates of the relative amount of antigen occurring in small rapidly diffusing bodies, and underestimates of relative antigen amounts in larger particles. At the level of approximation with which estimates of antigen abundance has been made in this section, such effects are not important. However, using raw assay values as a proportional measure of antigen abundance, as was done to produce quantitative sedimentation profiles of antigens in hnRNP (see figs 2, 3 and 7 of RISAU et al., 1983), would be expected to lead to distortion with respect to the true distribution of antigen. Luckily, these effects do not qualitatively alter any of the conclusions made in that study.

R.9.3. Detection of rare antigens, current achievements and future prospects

In section R.7.2.1.3 I showed that, assuming a lower level of signal detection of 10^3 cpm, the theoretical lower limit of antigen detection using a detector of the affinity currently available to us, at the concentration employed in the standard assay, is approximately 10^8 molecules. In IRMA, I argue that such a limit is attainable (R.9.2.2 see also R.7.3.4.1). In ELISA, 10^8 molecules can also be detected experimentally (R.8.3, P11). However, the ELISA results were obtained with very low signal levels in systems with low backgrounds. Using crude Drosophila nuclei to coat plate surfaces it is unlikely this level of sensitivity could be reached.

Under standard assay conditions using crude nuclear preparations, the protein from about 2 10^6 (20 µg Biuret, 10 µg Bradford) nuclei is used per assay as a source of antigen. However, the capacity of the solid phase (R.7.4.3) only allows the protein equivalent of 10^5 nuclei to adsorb per

microtitre plate well. Thus, if no preferential binding or exclusion of an antigenic protein occurs, and provided that the monoclonal antibody which identifies it is present in saturating amounts, the minimum detection limit for the protein will be:

$$\frac{10^8}{10^5} = 10^3 \text{ copies/nucleus}$$

However, as the results shown in sections R.7.4.1, R.7.4.2, R.8.2 and the previous section R.9.2.2 demonstrate, for some antigens exclusion effects almost certainly do occur and are of sufficiently high magnitude (> factor 10) to seriously affect the sensitivity of the assay for them. A logical consequence of exclusion in a closed system is that some other proteins must be preferentially adsorbed. The magnitude of exclusion could logically be either much greater or much less than the empirically observed factor 10. The maximum magnitude for preferential binding however is a factor 10 (20 fold excess of protein to binding sites, but the physical limit set by diffusion means that only half of this excess could bind under standard conditions). However, it does **not** follow that for any particular antigen preferential binding of this magnitude must take place: exclusion might be caused by the aggregate effect of many proteins in a mixture all binding preferentially by a very small amount. A low assay signal may therefore signal either a poorly affine monoclonal antibody, an authetically rare antigen or an antigen which adsorbs poorly from a mixture. Conversely some rare antigens (e.g. if of low M.W. and soluble in monomer form) might give rise to spuriously high signals.

Of the antigens I have examined to date, those which give the lowest signals which can reliably be distinguished from background are the P12 and V4 entities. Both give signals of about 800 cpm under standard assay conditions, corresponding to about 4000 cpm at saturation with second antibody. If

either of these monoclonals is sufficiently affine to saturate its antigen then, if transfer from the nuclear suspension used as antigen source to solid phase occurs at the bulk rate, this would correspond to a frequency of 10^3 - 2 10^3 copies per nucleus. Frequencies of this order would thus represent the lowest level of antigen which can currently be detected with the system. Either P12-ag or V4-ag might really occur at this low frequency (approximately 1 antigen molecule/5000 nucleosomes), but it would be uncalled for to take these estimates too seriously until the assumptions upon which the estimate is based, namely bulk rate adsorption and monoclonal antibody saturating antigen, have been tested. All the antigens for which these assumptions have been tested (P11, S5 and X4) and therefore for which any confidence can be placed in frequency estimates have copy numbers of about 10^5/nucleus (see previous section), but on the other hand deliver signals well above the minimum required for detection.

The currently attained lower level of detection for antigens using solid phase assay can thus be placed in the interval 10^3 - 10^5 copies/nucleus. This figure can almost certainly be improved. The following suggestions indicate some of the strategies which could be followed.

Firstly, nuclear extracts rather than crude nuclei could be used. For an equal amount of protein per well, the use of extracts would allow the number of nuclear equivalents of an antigen per well to be increased. If the fractions of some standard fractionation scheme were assayed in parallel using equal amounts of protein/assay, then not only would it be possible to lower the lower limit of detection, but also to gain information about an antigen's fractionation behaviour (e.g. salt extractable, soluble, insoluble etc.,). By using longer time periods for adsorption than at present (several hours, rather than a single hour) it would also be possible to reduce the amount of protein required to obtain saturation of

the binding capacity of the solid phase. Procedures to break up large particles (DNAase, RNAase, salt, reducing agents) would also be expected to increase transfer efficiency. By using amounts only just exceeding the capacity of the well surface, it is conceivable that exclusion (and preferential binding) effects could be reduced. This would allow more accurate comparison of relative antigen abundance.

This approach has been tested empirically and there is no doubt that it leads to improvements. Using the total hnRNP fraction (see fig. 43), for example, it was possible to increase the V4 signal by a factor 3.5 compared to that obtained from crude nuclei. Similar increases were obtained for most of the other class I antigens. The signal from the P12 antigen (a class II antigen, not present in hnRNP, for explanation of class I and class II see SAUMWEBER et al., 1980) could be improved by a factor of 2 to 5 by taking 400 mM salt extracts of nuclei, or the nuclear residue remaining after hnRNP preparation.

The above suggestions are confined to the "antigen" side of the assay. On the "antibody" side, there are also possible improvements that could be made, particularly with respect to the properties of detector, especially that used for IRMA. Most of these improvements could only be achieved as part of a long term program. The ideal high sensitivity IRMA detector might have the following characteristics:

1) Very high affinity ($>10^{11}$M) for all sites on monoclonal antibody. This might give as much as a factor 5 improvement.

2) Binding to as many sites as possible on the monoclonals. This might give as much as a factor 3 improvement.

3) Increased specific activity both by increased iodination: 3 moles I/mole IgG (factor 3 improvement) and possibly by the use of ^{131}I (also a factor 3 improvement).

4) It would be used at near saturating concentrations to give a maximal absolute response. The improvement in assay signal expected here is included in the improvement expected from high affinity.

Taken together, these improvements might allow a increase in sensitivity by a factor 50.

The same properties as those desired for an IRMA detector are also wanted for ELISA detectors. However, the commercially available antibodies which we use are not well characterised, and their "specific activity" is not known, so it is difficult to predict quantitatively how much improvement can be expected, especially since some, such as point 2 above (see R.7.4.5) may already have been implemented. However, there are other ways in which this assay might be improved such as the use of biotin-avidin "sandwich" technique. An even greater improvement, by several orders of magnitude, would be possible if a fluorigenic rather than a colorigenic substrate were used to detect coupled enzyme activity (ALI and ALI, 1983): fluorescent reaction products can be detected in picomolar concentrations rather than the nanomolar concentrations which set the limit for optical absorbance measurements of coloured substrates.

In addition to improving these detector characteristics it would be advantageous to work with concentrations of monoclonal antibody below 0.1 µg/ml (10^{-9}M). This would automatically ensure selection of high affinity antibodies and minimise background problems (cf R.7.3.1.2.3 and R.7.4.4).

Thus, it is conceivable that by combining antibody and antigen improvements that an increase in sensitivity of a factor of 100 or possibly even greater might be achieved. This would mean that antigens occurring at frequencies of as little as 10 molecules/nucleus would be detectable. Indeed in

IRMA, since this limit is based on the very conservative assumption that at least 1000 cpm is required to generate a detectable signal, it follows that if 100 cpm above background could reliably be measured, 1 molecule of antigen/nucleus might be be made visible.

RESULTS and DISCUSSION

PART IIB

Comparative Measurements of Drosophila Antigens

R.10 Relative Distribution of hnRNA associated antigens on polytene chromosomes of Drosohila melanogaster salivary glands determined by quantitative analysis of immunofluorescence images obtained by double direct immunofluorescence staining

R.10.1 Rationale

In principle, it must be possible to estimate the absolute amount of an antigen in a histological preparation by measuring the fluorescence intensity of fluorescently labelled antibody bound to it. However, even when the amount of fluorescence has been calibrated in terms of number of antibody molecules, there remain a number of problems.

i) The efficiency with which the antibody binds to antigen under the conditions of assay must be known. This will be determined both by the steric accessibility of antigen and (which may be very low, see for example SASS and BAUTZ 1982 and also BUSTIN 1978), as well as the intrinsic affinity of antibody for antigen. Obviously the simplest way of controlling binding efficiency determined by affinity is to attempt to saturate all available antigenic sites with antibody, however this may not always be possible (see below).

ii) If the sample is fixed, in addition to possible changes in steric accessibility the immune reactivity of antigen may actually be destroyed (cf. R.11.3), and this inactivated fraction must then also be estimated.

iii) Crossreactivity and/or non-specific binding of antibody must be kept to a minimum to be able to clearly localise the antigen containing region. This may require working with an antibody concentration below that required to saturate all antigenic sites.

iv) If indirect techniques are used the efficiency and amplification factor with which the labelled detector binds must also be determined.

The studies cited above and some of the observations made using solid phase assay suggest that these problems may not be

negligible. However no satisfactory attempts have yet been made to estimate their magnitude.

One way to gain information about the quantitative properties of antigens in biological systems, which sidesteps a number of these problems, is to restrict measurements to comparative ones. Pairs of antibodies are reacted simultaneously with antigen containing material which has been prepared in a standard fashion, but the state of the cells from which the antigen-containing preparations are made may vary. The ratio between the signal from the two antibodies then provides an indicator of the state of the biological system. Obviously, even this method can provide no safeguard against spatial heterogeneity within individual preparations, nor can it eliminate the danger of fixation artefacts, caused, for instance, by changes in the susceptibility to fixation. However, in as much as such changes may have a biological cause, artefacts may still be useful indicators of changes in system properties. It is also advisable to use saturating amounts of both antibodies to avoid differences which might be the trivial result of measuring binding at different positions on the binding curve.

The "ratio" principle was exploited to ask questions about the distribution and relative abundance of hnRNP-associated antigens on polytene chromosomes from the salivary glands of Drosophila melanogaster. To obtain a quantitative measure of immunofluorescence from a pair of monoclonal antibodies under exactly the same conditions of fixation, quantitative image analysis of the fluorescent images and direct immunofluorescence was employed. The images to be analysed were obtained by using the appropriate filters (M.6.1) to separate red and green fluorescence images from chromosome preparations which had been stained simultaneously by a pair of antibodies, one of which had been labelled with FITC, to fluoresce green, and the other with TRITC, to fluoresce red. The distribution of fluorescence intensity

along the length of the chromosomes was analysed using the methods outlined in M.6.2.

R.10.2 Estimation of antibody concentrations required to saturate direct immunofluorescence measurements.

Direct immunofluorescence was used because this was the simplest way to prepare a pair of discriminatory reagents. In order to estimate approximately the concentration of directly labelled antibody required to saturate antigen in fixed Drosophila material, quantitative image analysis of fixed Drosophila Kc cell nuclei was made at different concentrations of directly labelled antibody. To estimate whether the direct labelled antibody might in any way be inactivated, indirect fluorescence staining (using an anti-antibody fluorescing with another colour) was carried out on the same preparations and the signal level compared to that obtained using an identical concentration of unlabelled antibody.

Kc cell nuclei were chosen because they were expected to stain uniformly (in comparison to polytene chromosomes). It was assumed that they would not differ greatly from polytene chromosomes in their ability to bind antibody. As a measure of staining intensity, the mean fluorescence intensity per pixel (image point) within the nucleus was determined. For this purpose the "nucleus" was defined as an area including all pixels whose intensity value exceeded some empirically determined threshold level (=cytoplasmic staining); this threshold was varied slightly from one microscope slide to another but of course was kept constant when nuclei from the same slide were being compared.

The staining intensity of different nuclei within the same preparation was found to be uniform by this method and varied by approximately only +/- 10% about the mean. Table 55 shows a representative sample of the variabilty that was found. Not only was fluorescence intensity similar between

cells on the same slide, but was also similar on different slides (range +/- 10% of mean) when identically treated slides were examined using the same instrument settings (image amplification).

Fluorescence Intensity/ Pixel (arbitrary units)	Mean (95% confidence limits)
91.6	
74.3	
87.2	
83.9	
94.2	86.2 +/- 9.7
	(76.6 - 95.9)

Table 55 **Variation in fluorescence intensity between Kc nuclei stained by direct immunofluorescence.**
Fluorescence intensity values from 5 different Kc cell nuclei on the same microsope slide stained with TRITC-labelled T7/3/3/4 monoclonal antibody at a concentration of 30 µg/ml.

As a measure of the approach to saturation the ratio of fluorescence intensity at two different antibody concentrations differing by a factor of 10 was used. It was argued that if the fluorescence intensity ratio thus obtained was close to 10 this would indicate that the highest antibody concentration would still be far removed from that required to achieve saturation. If a low ratio was obtained this would indicate that saturation was being approached. As table 56 shows, increasing the concentration of either TRITC or FITC labelled T7 from 3 µg/ml to 30 µg/ml increases the fluorescence intensity of the stained nuclei by only a factor of about 2 indicating that at 30 µg/ml nearly all available T7 antigen will be bound by antibody. The increase in the signal from the P11 antibody was clearly larger (approx. a factor 6), suggesting that saturation might not yet have been reached for this antibody even at this high concentration. Still higher concentrations of this antibody (100 µg/ml) caused visible staining of the cytoplasm of the Kc cells which was interpreted as being non-specific binding. Non-specific

binding, mainly confined to the nucleus, may also be a cause of the apparent lack of saturation observed here (P11 is known from solid phase experiments to have a greater tendency than other antibodies to bind non-specifically see R.7.3.1.2.3, R.7.4.4.1). In any event, 30 µg/ml was subsequently used to stain polytene chromosomes, using the rationale that it represented a good compromise between obtaining saturation and avoiding gross levels of non-specific staining.

Antibody	Fluorescence label	Direct Immuno-fluorescence Ratio 30 µg/ml:3 µg/ml	Indirect Immuno-fluorescence Ratio 30 µg/ml:3 µg/ml
T7/3/3/4	TRITC	2.9 (2.2 - 4.1)	1.5 (1.1 - 2.2)
T7/3/3/4	FITC	1.9 (1.5 - 2.3)	1.2 (0.9 - 1.6)
P11/6/3	TRITC	5.6 (3.7 - 9.4)	1.9 (1.2 - 4.3)

Table 56 **30 µg/ml monoclonal antibody is sufficient to nearly saturate antibody binding to fixed Kc cell nuclei**

3 µg/ml or 30 µg/ml of the directly labelled monoclonal antibodies was bound to Drosophila Kc cell nuclei and the mean fluorescence intensity of at least 3 (usually 5) nuclei determined by quantitative image analysis. Second antibody at the concentration routinely used for indirect staining, but with the opposite fluorescence label, was used to stain all preparations indirectly. The values in brackets are the worst case limits of the ratio estimates (see R.9.1.2) based on the 95% confidence limits of the means from which the ratio values were calculated.

Table 56 also shows the fluorescence intensity ratios obtained by indirect staining of the directly stained preparations. Exactly the same cell nuclei were examined. In this case, the concentration pairs of all three antibodies (the P11 preparations especially) give lower ratio values than their directly stained counterparts. This result implies that the amount of second antibody is in some way limiting. Either the concentration of second antibody may be insufficient to saturate first antibody or, at high densities, a proportion of

the first antibody may be inaccessible to second antibody. In either case this result indicates that caution should be excercised before attempting to use indirect methods quantitatively. In particular, the question of whether some first antibody is inaccessible to second antibody should be clarified.

Quantitative image analysis revealed that the fluorescence intensity obtained from **indirectly** stained images, using the same amount of either unlabelled or fluorescently labelled (other colour) T7 or P11 antibodies to stain Kc cells, was similar (to within 20%, data not shown). This indicates that directly labelled mouse antibody was immunologically largely intact (both as antigen and antibody).

The concentrations of antibody apparently required to saturate chromosomal binding sites are higher than those that might be expected from solid phase assay experiments (cf. R.7.4.4.2). However the duration of exposure to monoclonal antibody, the surface topology of the antigen presenting object and the wash conditions are different. ARDNT-JOVIN et al., (1983) needed 30 µg/ml of a polyclonal anti-Z DNA antibody preparation with a mean K_{eq} of 10^8 M^{-1} to saturate Chironomus polytene chromosomes, but used a staining period of 5 hours, rather than the 45 min I used. It should be noted that under the saturation binding conditions used by these authors less than 1 in 500 of the antibody molecules with which their chromosomes were incubated bound antigen.

Because of difficulties in obtaining adequate machine time (it took two days to collect the data shown in table 56), and a number of other logistic problems, these measurements were not repeated with the other antibodies used for the direct immunofluorescence experiments reported in the rest of this chapter. On the basis of the experiments here the other antibodies were also used at a concentration of 30 µg/ml. That this concentration of directly labelled antibody was

sufficient to saturate the antigen on polytene chromosomes is therefore an assumption based on two premises: one, that the antibody binding characteristics of approximately 10^4 Kc cells (the number under a coverslip) and between 10 or 20 sets of polytene chromosomes is similar, the other that the binding characteristics of the S5 and X4 antibodies are similar to that of the T7 antibody.

Two polytene chromosomes from the same slide stained with 5 µg/ml of the S5 antibody were analysed. One gave results which were qualitatively and quantitatively indistinguishable from those using higher concentrations of antibody (not shown). The other showed a distribution which varied from that most frequently observed (see fig. 52e), but because the S5/X4 ratio was greater than average at one locus and less than usual at another, it was felt that these differences could be ascribed to the state of genetic activity of this particular chromosome (see R.10.6). For reasons explained in section 3.4.2, at most 2/3 of the IgG in any S5 preparation is likely to be able to bind antigen. 30 µg/ml and 5 µg/ml thus correspond to concentrations of 20 µg/ml and 3 µg/ml of active antibody respectively. The empirical results therefore suggest that, whether or not saturation had actually been reached at a concentration of 20 µg/ml active S5, the staining pattern **on polytene chromosomes** was not grossly affected within the range 3 - 20µg/ml. Nonetheless, it would clearly be desirable to have more convincing evidence that all the antibodies tested were being used under saturation conditions.

R.10.3 Maximum Intensity Profiles (MIPs), scatter plots and alignment of fluorescence images for quantitative comparison.

The pattern of quantitative variation in fluorescence intensity along the long axis of polytene chromosomes was recorded in the form of **Maximum Intensity Profiles** (MIPs, see M.6.2). In a MIP the intensity value of the pixel with the

highest fluorescence intensity with respect to the chromosome's **breadth**, is displayed for each pixel of the length of chromosome that is analysed (see figs 49 - 52 for examples of MIPs). The MIPS were extremely reproducible: providing care was taken not to mechanically disturb the object stage of the microscope, repeated MIPs of the same colour fluorescence from the same field of view of a particular preparation gave essentially identical profiles. Gathering the data for a signal-averaged, digitised image only took a few seconds, so exposure to high intensity illumination was minimal. Under the conditions of illumination which were usually used, and in the presence of p-phenylenediamine, bleaching was negligible (<1%) until at least 3 MIPs had been recorded.

According to the late R.BURBERG, the approach of constructing MIPs is legitimate because the profile of fluorescence intensity with respect to the breadth of the chromosome is of the broad form shown in fig. 47a. The maximum of this distribution can be found with little difficulty, and only a small error will result if the actual maximum does not fall exactly inside the region of the chromosome's breadth which is sampled in the search for the maximum (fig. 47b). The MIP is therefore a measure of the relative brightness of antibody staining at a set of points, and as such is a measure of the **concentration** of antigen at these particular positions on the chromosome. Because the profile of fluorescence intensity with respect to chromosome breadth is relatively uniform this concentration is also representative of the concentration of antigen at that particular locus. However, since "bands", "interbands" and "puffs" can vary greatly in their physical dimensions, both laterally and longditudinally, the **amount** of antigen at loci exhibiting the same maximum concentration (MIP value) may not necessarily be the same.

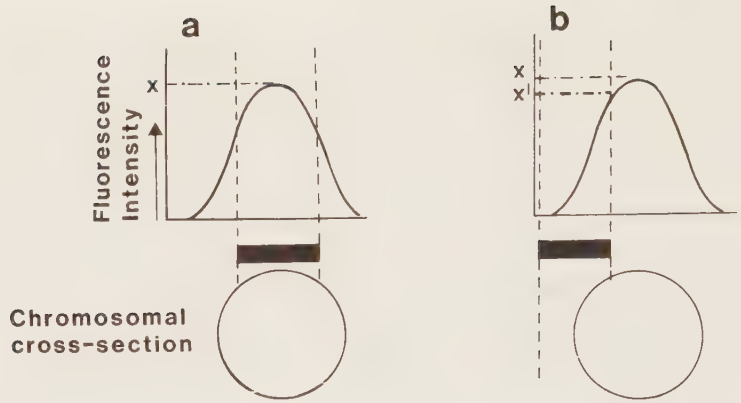


Fig 47 **Assumed distribution of fluorescence intensity, with respect to breadth, of an immunofluorescently stained polytene chromosome.**

a) The assumed profile: search region for maximum intensity (region between the ends of the heavy bar) has been ideally positioned by the investigator.

b) Same as a), but showing the effect of positioning the search region for X incorrectly: X' is recorded as maximum value rather than X but, unless the search region positioned by the operator is wildly misplaced, the proportional error X - X'/X is small because of the flattened form of the intensity profile.

For exact comparisons, it would be desirable to have a measure correlated to the **total amount** of antigen, as well as its concentration. This would require measuring the integral of the fluorescence intensity curve with respect to the width of the chromosome for each pixel of the length of chromosome sampled. This approached was not realised for two main reasons (pers. comm. from the late R. BURBERG). Firstly it was difficult to find an algorithm which was able to adequately define the edge of the chromosome on the basis of the fluorescence intensity alone (where is the edge of a region which is dark on a dark background?). Combining information from phase contrast and fluorescence images should, in principle, be able to solve this approach, but was not attempted because of the second problem. Namely, secondly, the data processing requirements of adopting the integral approach

are much greater than when the MIP approach is used. Because of limitations in the amount of memory available for computation and because of limited computational speed an increased data processing demand would have severely limited the length of chromosome which could be analysed at one time.

Using MIPs from the same object obtained by staining with two different antibodies, variation in the relative concentrations of two different antigens with respect to position in the chromosome could now be made. However, for this approach to be valid, it is a necessary precondition that the two MIPs are exactly aligned (i.e. the fluorescence images from which they are obtained must be superimposable).

Unfortunately, a minor defect in the Axiomat microscope (almost certainly a slight mechanical imprecision in the position at which the movable fluorescence filter holder became engaged in its placement slot), resulted in a shift between the red and the green images, on whatever medium they were displayed (monitor screen, printer) of between 1 and 3 pixels. With respect to the total breadth of the field of view this misalignment is small, only 0.25% to 0.75% of the width of the observed field. However, at the scale of the smallest distinguishable features on the chromosome, narrow interbands about 10 - 15 pixels wide, such a displacement might be very misleading. No misalignment was observed in the vertical axis.

Each pair of images generated a large amount of data, more than 200 intensity values per image, and the exact amount of misalignment varied from one pair of images to the next, so it was not possible to realign the data by making a standard correction. Furthermore it was often difficult to judge the misalignent of data by simple inspection, because there might be additional, authentic, differences between the two sets of data (see R.10.5) and it was not always easy to distinguish between the two sources of discrepancy.

An objective way to align the MIPs was found by making use of scatter plots, in which the fluorescence intensity at a

particular length coordinate from the MIP of one colour was plotted against the fluorescence intensity of the other MIP at the same coordinate. If two identical images were in register, such a plot should give rise to a straight line. If they are out of register the data will scatter about this straight line (if the reader doubts me he/she can try plotting the sequence 1,2,3,4,5,4,3,3,1 against itself, against 0,1,2,3,4,5,4,3,3 or 2,3,4,5,4,3,3,1,0 and see what happens). This condition should also be true for corresponding data sets in which only the relative amplitude, but not the position, of signal generating regions is different, although in this case scatter will be centered on a set of straight lines of different slope. It follows, that if the coordinates of one set of data are systematically shifted with respect to the coordinates of the other, the resulting scatter plot which gives the minimum scatter between the two data sets should indicate which shift gives the correct alignment.

Inspection of fig. 48 shows that this method was effective in practice. The scatter in panel c), in which the each red fluorescence data point has been shifted one pixel to the left with respect to the green fluorescence data points, is clearly less than the scatter which results from plotting the unshifted data, (panel a). Shifting the red data a further point leftward, however, clearly makes the scatter larger again (panel d). Shifting the red data rightward by one pixel increases the scatter compared to the original (panel b).

Using such an approach the data from the ends of the MIPs becomes lost but this loss is negligible compared to the total amount of data gathered.

R.10.4 Comparison of MIPs: is the method valid?

The validity of comparing MIPs can be tested by simultaneously staining a chromosome preparation with the red and green fluorescing versions of the **same** antibody. If MIPs

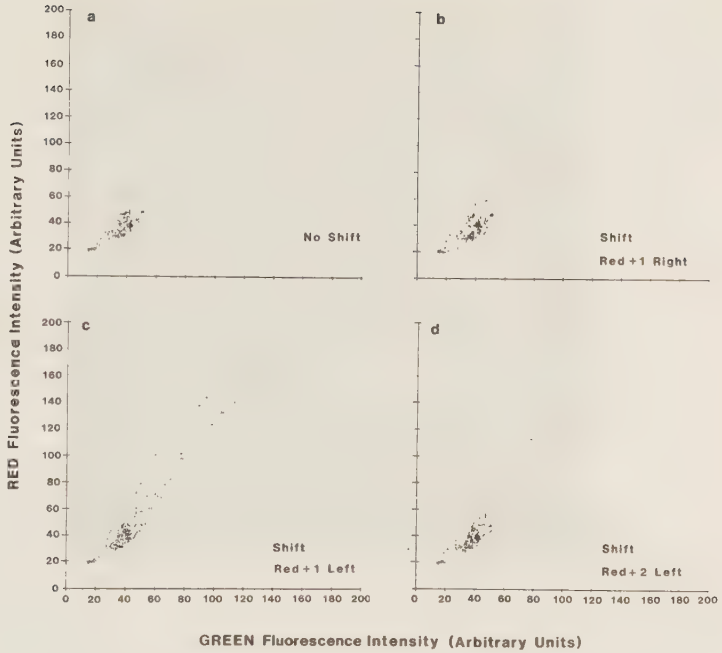
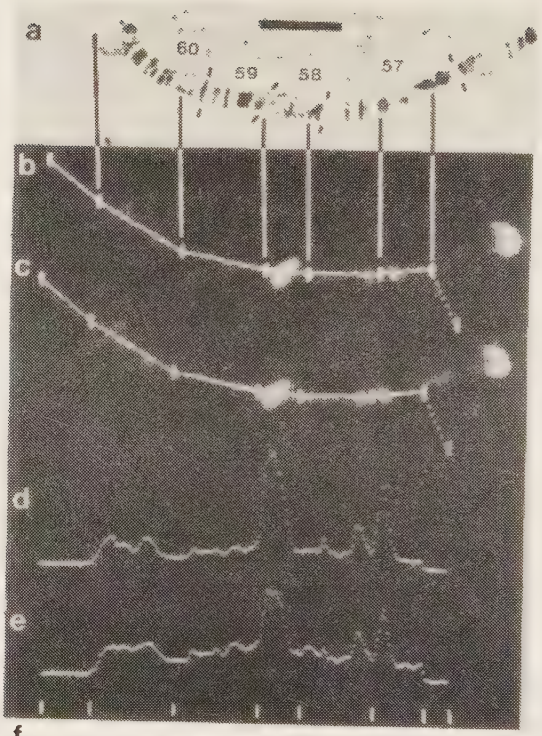


Fig. 48 **Alignment of MIPs by use of scatter plots**

Scatter plots, were constructed as described in M.6.2. The original data consisted of two sets of fluorescence intensity values (Ng1,Ng2...Ngn) from the green image and (Nr1,Nr2..Nrn) from the red image. In panel a) each Nr(x) value is plotted against the corresponding value Ng(x). In panels b), c) and d), the same data set has been used but each value Nr(x) has been plotted against either Ng(x+1), Ng(x-1) or Ng(x-2) respectively. The small numbers on the plot indicate that that number of data points occurred at that coordinate. Data from the MIPs shown in fig. 50c have been used; green fluorescence from X4-FITC, red fluorescence from X5-TRITC.



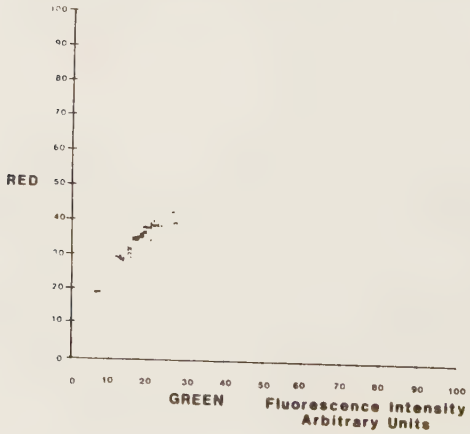


Fig. 49

(see next page for legend)

give an authentic reflection of the concentration of an antigen at different positions on the chromosome, and if direct labelling does not interfere with either the specificity or the affinity of antibody binding, then the pattern of intensity peaks in the two resultant MIPs should be identical. The amplitudes need not be identical, because intensity units are arbitrary and illumination conditions were only chosen to give intensity values which roughly corresponded. The background signal using each of the differently labelled antibodies might also be somewhat different. The ratio between the amplitude of the peaks in the two MIPs, adjusted for background fluorescence, should, however, be constant. This should be reflected by a linear scatter plot, whose slope is determined by the relative amplitude of the fluorescence peaks.

The data presented in fig. 49 show that these

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Fig 49 **Red and green fluorescence from polytene chromosomes stained with a 1:1 mixture of TRITC- and FITC- labelled derivatives of the same antibody give identical MIPs.**
The chromosome preparation was stained with S5 antibody labelled with TRITC (0.3 mol/mol) or FITC (2.7 mol/mol) at a concentration of 30 µg/ml each. The MIPs and digitised fluorescent images were aligned using the "minimum scatter" procedure outlined in the previous section (R.10.3). Other details as described in M.6.2.
a) phase contrast image of the chromosome (tip of 2R) horizontal bar represents 10 µ, b) and c) digitised image (black and white representation) of red and green fluorescence respectively photographed from monitor screen. The thick vertical bars indicate the width of the 9 pixel wide strip which was searched for maximum intensity. The dashed line, approximately following the contour of the chromosome, marks the path which the mid-point of this strip followed. d) and e) MIPs of red and green images respectively. The individual points on the photographs of the MIPs made from the monitor screen do not necessarily correspond to individual pixels. The focus of the electron beam is too coarse and the control of its angular deflection too imprecise to allow neighbouring pixels to be resolved (they appear fused). The low resolution is a result of design inadequacies in the coils used to control the path of the electron beam and is a property of the colour monitor used for this work and not of the digitised signal. On a black and white monitor with better resolution more points would be observed. f) scatter plot of the data in d) and e).

expectations are fulfilled. Panels b and c of fig. 49 show the digitised fluorescence images of the tip of a chromosome 2R originating from TRITC and FITC labelled S5-ab with which it was simultaneously stained. The two images appear very similar and this is confirmed by their respective MIPs, shown in panels d and e. The scatter plot shown in panel f reveals that, except for a few data points at the lowest and highest intensities measured, all the points are tightly clustered in a single distribution approximating a straight line with a slope of 0.73, i.e. the fluorescence intensity measured from the TRITC image (corrected for background) had values only about three quarters those from the image from the FITC labelled S5. The line does not regress to the origin, but is offset somewhat along the ordinate. This offset represents a higher background fluorescence from areas outside the chromosome observed using red rather than green fluorescence. The origin of the difference is unknown.

There can thus be little doubt that the comparison of MIPs is should provide a reliable method for finding similarities and differences in the distribution of different antigens.

R.10.5 Quantitative comparison of relative staining by different antibodies

Having established that MIPs give a reliable and reproducible measure of the concentrations of antigens at different positions on the chromosome, it is now possible to ask whether the relative distribution of antigens is the same or different. The analysis of MIPs from the tips of the 2R, 3L, 3R and X chromosome arms indicates that the distribution pattern of the 4 hnRNP-associated antigens tested appears to fall into two groups: one containing the S5-ag, P11-ag and X4-ag antigens, the other T7-ag.

The P11, S5 and X4 antigens vary by a factor of 10 or more in the relative concentration in which they occur in

different interbands. Only a subset of all interbands and puffs (<25%) contain concentrations of these antigens sufficiently high to give rise to prominent staining by fluorescent antibody (cf. fig. 52). In most of the interbands which are prominently stained, the concentration of each of the antigens appears to be quantitatively correlated. Fig. 50a - c shows some examples of this correlation; further examples can be seen in fig. 52b and c. The scatter plots in fig. 50h,i show that there are minor differences in the ratio between the two antigens, reflected in the somewhat greater spread of the points than when the distribution of an antigen is compared with itself (compare to the scatter plot in fig. 49), but to a first approximation the points in the scatter plot can be considered to lie on the same diagonal.

Although a quantitative correlation between pairs of antigens at an approximately constant ratio is the condition most frequently encountered condition (>80% of all prominently stained puffs and interbands), there are some clear exceptions. Departures from the most common ratio in both directions are found.

Fig. 50d shows an example of an interband in which the relative concentration of X4 is greater in one prominent interband (indicated by an arrow) than in the others. This is clearly shown in the scatter plot (fig. 50j) in which the data clusters around two diagonals of different slope: the diagonal of lower slope representing the data from the large X4 MIP peak.

Fig 50e shows an example of the opposite effect in which the concentration of S5-ag relative to X4-ag is higher in one interband than all the others. This is not easy to see in the MIP itself because the stained interband is not fully resolved as a peak, but rather as a broadened form of the peak at the extreme left of the MIP (arrow in figure). The discrepant ratio is seen more clearly in the scatter plot (fig. 50k), in which it gives rise to a diagonal of greater slope than the rest of the data. Its cytological counterpart is a region of

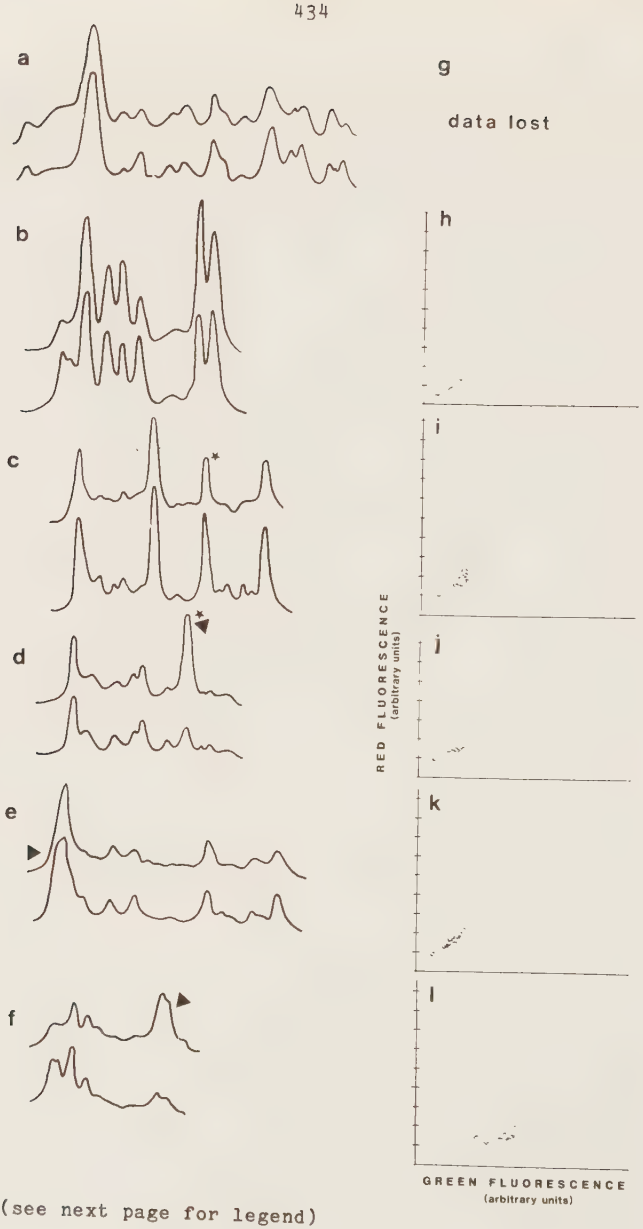

Fig. 50 (see next page for legend)

bright fluorescence at the very tip of the chromosome. Another example of an interband in which the S5/X4 ratio exceeds that in its neighbours is at the the tip of chromosome 3L (fig. 52b,d,e).

In the extreme example shown in fig. 50f, deviations in both directions occur in the same short stretch of chromosome (the MIPs are presented again in fig. 52 to show that this pattern is not representative). The S5 concentration in the peak at the left hand end of the S5 MIP is clearly greater

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Fig. 50 **Similarities (and differences) between the distribution of the antigens P11, S5 and X4**

Panels a-f, MIPs of antibody pairs staining the chromosome ends indicated in the table below. Panels h-l scatter plots corresponding to MIP pairs b-e.

Panel	Chromosome arm	Upper trace (FITC)	Lower trace (TRITC)
a	X	P11	S5
b	3L	X4	S5
c	2R	X4	S5
d	2R	X4	S5
e	3R	X4	S5
f	3L	X4	S5

All preparations were stained with 30 µg/ml of each antibody, except the preparation shown in panel f, where the S5 concentration was only 5 µg/ml. Antibodies were labelled (figures in brackets indicate mole fluorochrome/mole IgG ratios) as follows: S5-TRITC (0.3), X4-FITC (2.7), P11-FITC (1.6). MIPs were obtained by positioning the line along which maximum intensities were sought exactly as in fig. 49: the left hand end is always oriented to the tip of the chromosome and the extreme left- and right-hand end of each profile show fluorescence background (microscope slide unoccupied by any visible object).

Scatter plots were aligned as outlined in R.10.3. Numerical values have been deliberately omitted from the fluorescence intensity axes of the scatter plots, because only the pattern of scatter is relevant to the discussion in the text. The units used to measure intensity, were arbitrary and varied from experiment to experiment (the results shown are from five different experiments performed on different days, as much as a month apart) to a different extent for each colour of fluorescence (see M.6.5): hence differences in the slope of diagonals **between** different panels have no significance for the evaluation of antigen ratios.

than in its X4 counterpart, whereas the double peak indicated by the arrow is clearly greater in the X4 trace. This is reflected in the scatter plot (fig. 501) by a large amount of scatter, but nonetheless the data appears to be clustered around diagonals of only 3 different slopes, perhaps indicating that only a discrete number of S5/X4 ratios is possible.

Deviation of the S5/X4 ratio from the most commonly found one does not appear to be solely a property of the location on the chromosome at which it occurs. When different examples of a particular locus on the chromosomes from different larvae are analysed, most are found to be stained at the most commonly found ratio. Only a few examples are stained at the altered ratio. i.e. the ratio of S5/X4 at a particular locus (and presumably also the P11/(X4 or S5) ratio) is variable. An example of this can be seen in fig. 52, in which the S5/X4 ratio of the double MIP peak corresponding to the interband pair at map unit 62E and 62F is approximately similar to the S5/X4 ratio at the interband at map unit 61C in 3 cases out of 4 (fig. 52b,c,d) but in the other case it is clearly lower (fig. 52e). A further example of this variability occurs at the puffed locus 58DE on chromosome 2R (bulge at left hand end of map unit 58 in the phase contrast image of 2R shown in fig. 49a: compare the MIP peaks marked with the star in fig. 50c and d).

The rules governing the appearance of the T7 antigen appear to be different: even neglecting a high "background" level of staining along the entire chromosome, the T7 antigen appears to be present in a larger subset of interbands than that in which the antigens of the other group are found. This is shown in fig. 51a and b. The subsets clearly overlap, but even when T7 and the other antigens occur together in the same interband, there appears to be little quantitative correlation between them. The scatter plots fig 51c and d reflect these features: the lower edge of the area containing the scattered

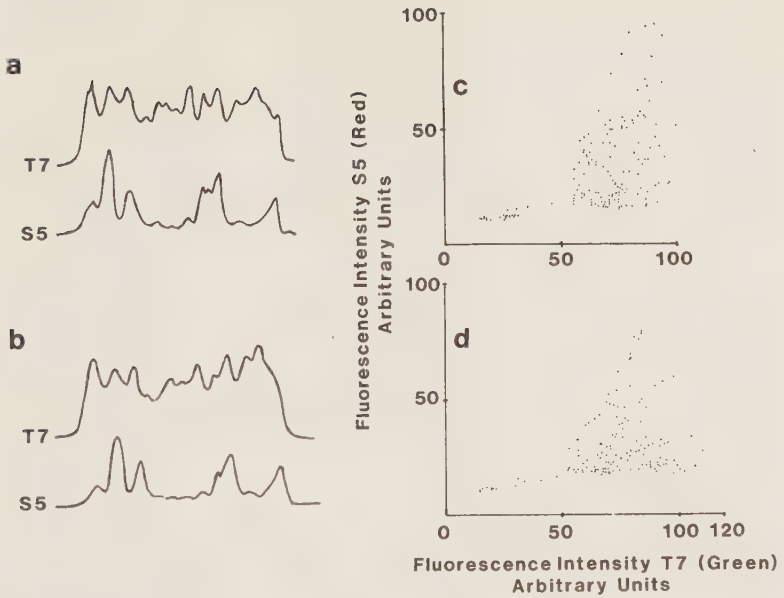

Fig. 51 **Differences in the distribution of the antigens T7 and S5** Panels a and b, MIPs from this antibody pair staining two different chromosomes. The upper trace in each panel is intensity of staining by T7-ab and the lower staining by S5-ab. In each case the tip of the chromosome arm 3L has been stained. Panels c and d, scatter plots corresponding to c and d. S5-TRITC labelled at 0.3mole/mole IgG, T7-FITC at 3.2 mole/mole IgG. Both chromosomes were examined in the course of a single experiment, using a squash preparation from a single larva.

points runs approximately parallel to the abscissa. This region of the plot corresponds to regions of the chromosome where T7 is present but S5 (and presumably X4 and P11) is essentially undetectable (?absent). No such region is found in any of the scatter plots show in fig. 50. This region extends to the highest green fluorescence intensity values measured, indicating that even the highest concentrations of T7 which

are found can occur independently of the S5 antigen. The converse is not true: S5-ag occurring in the near-absence of T7-ag would give rise to a set of points running parallel to the ordinate, but this is not found. Instead there is a maximum S5/T7 ratio marked by the diagonally running upper border of the area within which the data points scatter. The data within this area is widely scattered, indicating a wide range of possible quantitative relationships between S5 and T7. There does appear to be some clustering of data, but it is not as marked as that observed in even the most scattered of the S5/X4 plots. Variation in the co-occurrence of T7-ag and the other antigens was not examined directly.

In summary, the observed distribution of the S5,X4 and P11 antigens is both qualitatively and quantitatively (in the sense that a constant proportion between the antigens is maintained) similar in approximately 80% of the loci at which they occur. In the remaining loci either X4 or S5 may be present in excess of the other (the behaviour of P11 is presumably similar). The ratio of S5/X4 at a particular locus varies between chromosomes from different larvae. In contrast, the concentration of T7 antigen found in puffs and interbands is both qualitatively and quantitatively independent of the occurrence of S5/X4/P11 antigen set, although the two sets overlap. T7 can occur in the absence of S5/X4/P11 but not vice-versa.

These observations are fully in accordance with those made by KABISH (1982), using double label immunofluorescence staining (a combination of direct and indirect technique). He noted that the distribution of the S5 and P11 antigens was qualitatively identical (his methodology did not allow quantitative relationships to be reliably estimated) but that there were numerous qualitative differences between the distribution of the T7 and P11 antigens. On the basis of his observations the antigens T29, Q14, Q16 and Q18 would also be expected to behave similarly to the S5 group.

R.10.5.1 Quantitative comparisons: an interpretation

These observations suggest the following two hypotheses.

1) Whatever function the T7 antigen exerts it is probable that it can do so independently of the functions exerted by the other antigens. The converse may not be true: the function of the T7 antigen may be required for the action of S5, X4 or P11.

2) Under normal physiological conditions, the function of the S5/X4/P11 antigens, and probably a number of other antigens (see above) is coordinated. A constant proportion of each antigen might either mean that they form a molecular complex with one another, or it might mean that the hnRNA molecules with which they are known to be associated (RISAU et al., 1983) only have a limited number of association sites for each of them. I argue in the next section that the occurrence of different concentrations of antigen at a particular locus in chromosomes from different larvae is probably the result of normal developmental changes. Changes in antigen concentration and of antigen ratio are therefore probably reflections of chromosome dynamics during development and probably reflect either genetic activation or inactivation. If this is the case, the less common S5/X4/P11 antigen ratios might represent assembly or disassembly intermediates of the complexes in which these antigens can be isolated (RISAU et al., 1983), whereas the usual ratio represents the steady state.

There is evidence for assembly and disassembly processes involving these antigens. After heat shock the distribution of the antigens alters and that of P11-ag becomes qualitatively different from that from that of S5-ag (i.e. P11-ag is totally absent from loci where S5-ag is still present) (DANGLI 1983), so a disassembly process which separates this pair of antigens is clearly capabable of taking place. By the same token, assembly from separated components must also be possible,

since the original distribution of P11 antigen is restored following recovery from heat shock (DANGLI 1983, DANGLI and BAUTZ 1983).

These ideas are, in part, testable. If the T7 antigen is essential for post-transcriptional activity at most loci, microinjection of anti-T7 into the nucleus might inhibit the export of processed RNA from the nucleus. Microinjection of the S5, X4 or P11 antibodies, on the other hand, might be expected to affect only those RNA molecules subject to processing, and thus some mRNA might still be made and exported. mRNA from loci where S5, X4 or P11 antigens are found, in which processing is known to occur and for which cloned DNA intron probes are available, could be tested for any alterations in splicing after micro-injection. Less technically demanding, double direct immunofluorescence coupled to the use of staged larvae, or ecdysone induced *in vitro* salivary gland cultures (ASHBURNER 1972b), should be able to establish the proportions in which the S5,X4 and P11 antigens occur relative to one another during the initiation and termination phases of transcription at developmentally controlled loci.

R.10.6 Differerences in staining pattern between chromosomes from different animals

The chromosomes from different cells in the same preparation usually produced MIPs which were similar. The MIPs from the end of the 3L chromosome shown in fig. 51 are an example. Differences were however also seen, but are not unexpected (see below). Chromosomes prepared from larvae on different days for different experiments were also able to produce MIPs which looked remarkably similar (e.g. the MIPs in fig. 52c and 52f; the MIP shown in fig. 52f is also shown in fig. 51b, it has been repeated in another context for the sake of comparison). However differences which were sometimes quite large could be found between chromosomes prepared from different animals. This is shown in fig. 52 in which MIPs from

the tip of the 3L chromosome arm prepared from 5 different animals prepared on five different days are displayed. The interband at map unit 61F, for instance, varies greatly in the intensity with which it is stained by both the S5 and the X4 antibodies. In fig. 52d for example, this band is prominently stained, whereas in all the other preparations, although it is detectable, it is only weakly stained (cf. also fig. 50b and fig. 51a).

The staining pattern at the tip of chromosome 2R by S5-ab is also variable. Comparison of the MIPs in fig. 49d,e with the lower trace in fig. 50c and fig. 50d shows marked differences. In fig 49d,e the most prominent peak of fluorescence, in the centre of the MIP, comes from puff 58DE. In fig. 50c this locus also shows prominent fluorescence, but there are two additional prominent fluorescence peaks to its left (loci 59F and 60EF). In fig. 50d locus 60EF is giving rise to the most prominent fluorescence, and the S5 derived fluorescence both from the 58DE and 59F loci is markedly reduced.

In part some of these differences may come from differences in the degree to which the chromosomes are stretched during preparation. In fig. 52c the length of the MIP between the tip of the chromosome and the stained peak arising from interband 62F is clearly longer than that from other preparations. However, it is not clear whether this is entirely an artefact of preparation. Despite the extension of the tip region, the distance between the fluorescence peaks from interbands 62F and 63E in fig. 52c is similar to that found for the preparation shown in fig 52b. Probably a part of the variation in stretching can be attributed to the mechanical properties of particular regions of the chromosome, which may change during development.

The difference in antibody staining pattern between chromosomes obtained from different animals should come as no surprise. The larvae from which they prepared were unstaged, that is to say their age was uncertain, but they were usually

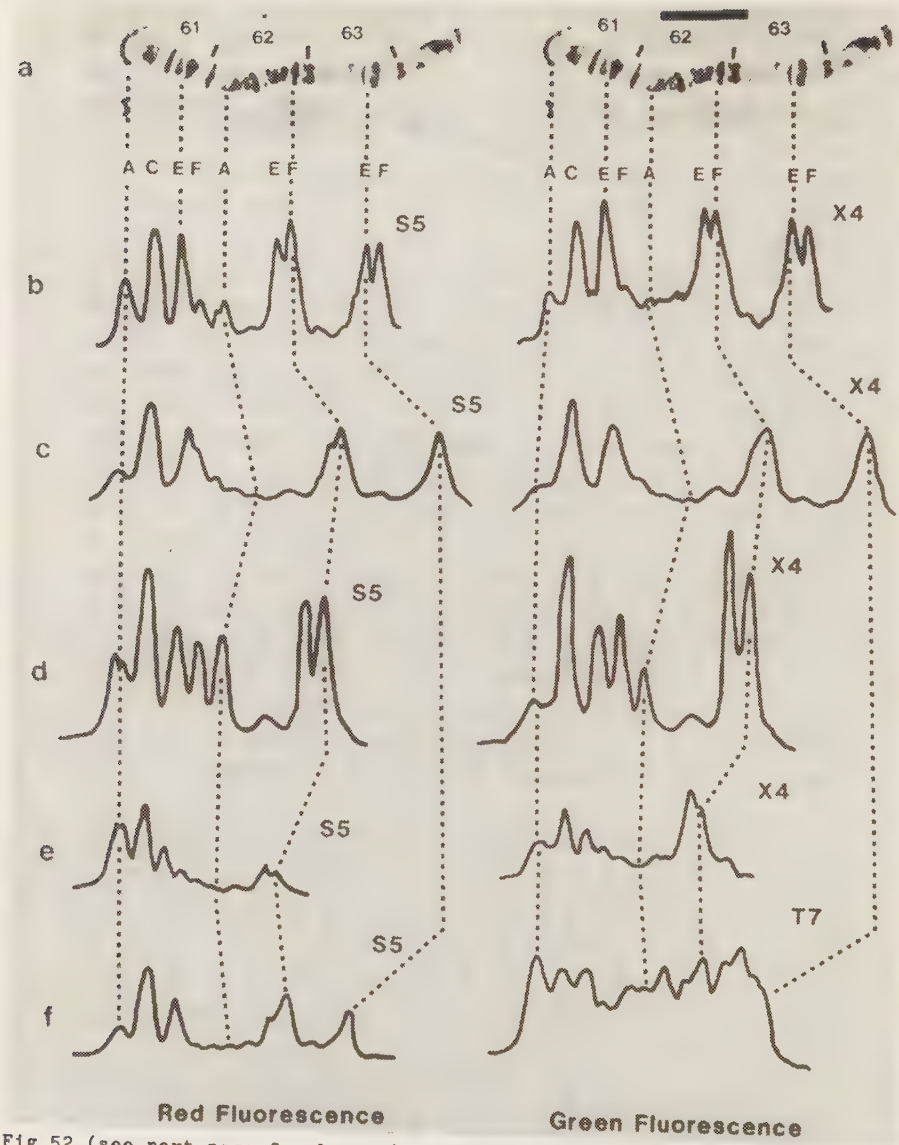

Fig 52 (see next page for legend)

taken between the time that they had crawled out of their "brei" and before they had pupated, which act they perform by crawling up the vessel wall and attaching to it, by means of their salivary secretion, before they metamorphose. This period lasts about 12 hours and it is known that a large number of chromosomal loci undergo changes in the state of their "puffing" during this period (BECKER 1959; ASHBURNER 1972a, KRESS 1972). Since puffing is usually asociated with changes in genetic activity (transcription) (PELLING 1959, 1972, MUKHERJEE and BEERMAN 1965, ZHIMULEV et al., 1981), variation in transcriptional activity at the loci concerned is the most likely explanation of the variation in MIP patterns between different larvae. If might be possible to test this interpretation immunologically by double direct immuno-fluorescence staining using antibodies against hnRNP associated antigens in combination with antibodies against RNA-DNA hybrids (BÜSEN et al., 1982). A small amount of variation **within** preparations, i.e. between chromosomes from the same gland, may be attributable to small differences in puffing in different regions of the gland (BECKER 1959, see his fig. 9).

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Fig. 52 **Variation in MIP pattern obtained from the same chromosome of different larvae**

The tip of chromosome 3L has been analysed. Panel a: phase contrast image of chromosome giving rise to the MIP in panel b; bar = 10µ. Left hand side of figure panels b-f staining by S5. Right hand side of figure panels b-e staining by X4. Staining conditions as indicated in figs 50 and 51.

RESULTS and DISCUSSION

PART IIC

Investigation of Antigen Function

R.11 Immunological properties of Histone 1

This chapter documents some properties of antibody reactions with, which may provide information about the structure and function of this molecule. The possible position of the antigenic determinants against which different anti-H1 antibodies are directed (R.11.1), an immunological response which discriminates H1 bound to supercoils (R.11.2) and the sensitivity of the antibody-antigen reaction to chemical fixation of H1 (R.11.3) are reported.

R.11.1 Interspecific immunological crossreactivity of Histone 1

The Western Blot presented in fig. 53 shows that the nuclei of human, hen, calf, Drosophila and Chironomus cells contain a protein that crossreacts with the anti-calf thymus H1 antibody preparation 402.

The purified calf thymus H1 used as a standard for the blot (lane a) gives rise to a double band, as it does in gels stained with coomassie blue (not shown, but see e.g. LÖFFLER 1978). This heterogeneity appears to be natural and not to be the result of degradation. Its cause is unknown. The crossreacting protein from human cell nuclei (lane c) and the upper of the two bands from chicken erythrocyte nuclei (lane b) have an electrophoretic mobility very similar to the upper of the two calf thymus bands. The signal from the Chironomus nuclei (lane f) is weak and there appears to be more than one component, but the major band also has the same electrophoretic mobility as the least mobile calf thymus polypeptide.

The electrophoretic mobility of H1 from the above species is known to be very similar to that of calf thymus H1. The simplest interpretation of the above observations is therefore that the 402 antibody preparation is crossreacting with the H1 histone from these species.

Fig. 53 **Western Blot of histone 1 containing fractions from different species with a rabbit anti-calf thymus H1 antibody preparation**

Transfer of proteins to nitrocellulose sheets was as described in M.2.5.3. The blot was reacted overnight with 30 µg of the 402 anti-H1 preparation (R.4.1., table 16) in 5 ml 1% FCS in DPBS. All subsequent steps were carried out in DPBS containing an additional 0.8 M NaCl. Bound antibody was tagged by incubating with 1.5 10^6 cpm ^{125}I Protein A. The wash steps after the protein A step contained 0.1% Triton. The autoradiogram was exposed for 48 hr using Kodak X-Omat and a Quanta II intensifying screen. The poor quality of the autoradiogram results in part from overexposure and in part from an accident in the developer bath.

The samples applied to each lane of the 12.5% SDS polyacrylamide gel from which the blot was made were as follows:

a) Calf thymus Histone 1 (10 µg)
b) Chick erythrocyte nuclei (1 $O.D._{260}$ unit, approx. 50 µg DNA)
c) HeLa cell nuclei (" ")
d) Drosophila salivary gland nuclei
e) Drosophila H1
f) Chironomus salivary gland nuclei
g) Calf thymus Histone 1 (only the very edge of the lane has been transferred)

The Drosophila, Chironomus and chick preparations were kind gifts of M.RENZ. HeLa nuclei were a kind gift of E.SPINDLER. The amount of salivary gland nuclei applied is no known exactly. The radioactively labelled smear in the space between lanes a and b is results from overloading the gel with a preparation of calf lymphocyte nuclei containing degraded H1. The arrows at the side of the figure mark the position of the upper calf thymus H1 band on the blot.

Drosophila H1 is known to migrate more slowly than calf thymus H1 in SDS-PAGE (my own observations, not shown). Thus the position of the immunoreactive band from a sample of purified Drosophila H1 (lane e,) and of the uppermost band obtained from Drosophila nuclei (lane d) is also entirely consistent with the interpretation that it is the histone 1 from this species that is reacting with the 402 antibody. The lower of the stained bands from the Drosophila salivary gland nuclei may be either a degradation product or a tissue specific H1 related protein. Only a single band was observed in blots made from the nuclei of Kc cells (not shown).

The histone 1 in isolated chick erythrocyte nuclei is very much less susceptible to proteolytic degradation than that in the nuclei obtained from other tissues. It is therefore unlikely that the lower band in the blot obtained from chicken erythrocyte nuclei (lane b) is a degradation product of H1. It is much more probable that this staining results from the binding of the 402 antibody to histone 5 (H5), an H1 related protein which is present in chick erythrocyte nuclei in large amounts (NEELIN et al., 1964) and is known to migrate more rapidly than H1 in SDS-PAGE (cf. e.g. LÖFFLER 1978, his fig. 9).

Fig. 54 shows that the cross reaction with Drosophila H1 by the 402 antibody preparation could be confirmed by solid phase IRMA of whole Drosophila nuclei, although the conditions of binding H1 to the solid phase were critical for obtaining good results (cf. R.7.4.2). In addition fig. 54 shows that whereas the 147 preparation was also able to cross react with Drosophila H1, the 405/6 and 401/7/9 preparations showed little or no crossreaction. The crossreactivity properties of the 405/6 and 147 preparations could be confirmed by Western blotting (not shown).

There are two major conclusions to be drawn from these findings, one concerning the nature of antibody and the other the nature of antigen.

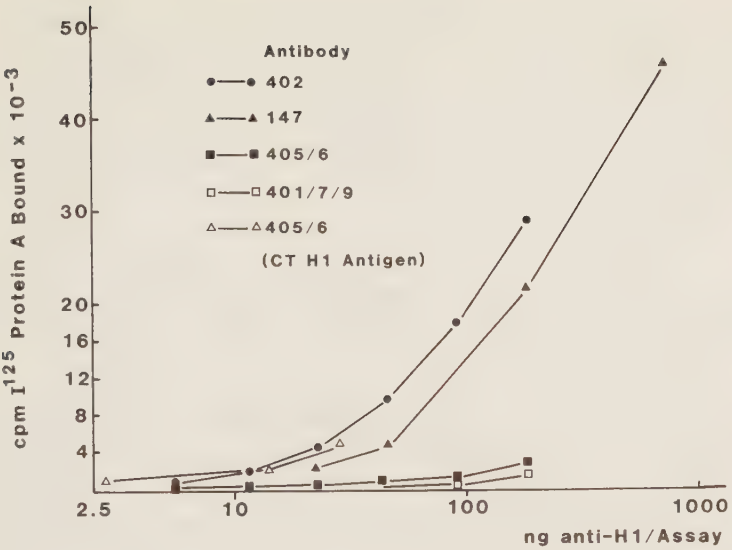

Fig. 54 **IRMA titration of crossreaction of Rabbit anti-calf thymus histone 1 antibodies with Drosophila nuclear Protein**

PVC microtitre plates were coated for 2 hours on ice with 100 µl of a Drosophila nuclear preparation (1 $O.D._{260}$ /ml) extensively sonicated in 2 M NaCl. The amounts of antibody indicated by the scale on the abscissa (note log scale) were reacted with antigen. Bound antibody was detected using 8.3 10^4 cpm of ^{125}I Protein A. Other assay details are as described in M.2.5.2.1. Only single values were measured. The symbols corresponding to each of the four antibody preparations tested are indicated at the upper left of the figure (see table 16, R.4.1 for details of preparations). The binding of the 405/406 preparation to a well coated using 100 ng calf thymus H1 is also shown. This control demonstrates that the 405/406 preparation was immunologically active.

Firstly the **difference** in crossreactivity to Drosophila (and ? other species?) between the 405/6 preparation and the 147 and 402 preparations indicates that at least two different antigenic determinants must be recognisable on the H1 molecule. Furthermore the proportion of antibody directed against these determinants can be very different in different antisera.

Secondly, the interspecific crossreactivity, especially the crossreactivity with H5, strongly suggests that the crossreacting antibodies are binding to the central region of the molecule (residues 22-100). This region of H5 and its counterpart region in the H1 molecule (residues 36-121) are known to have strongly conserved sequences (ALLAN et al., 1980).

Antibodies produced against calf thymus histone 1 by other investigators have exhibited properties which overlap with those described here.

Immunological crossreactivity between H1s from different species has been observed previously (BUSTIN and STOLLAR 1972, 1973a). In these studies, only the crossreaction between different vertebrate species was investigated. Cross reaction was extensive, but there were quantitative differences between species. BUSTIN et al., (1977) also compared the crossreactivity of an anti-calf thymus H1 preparation and anti-calf thymus core histone antibodies for crossreaction with their counterpart antigens in Drosophila. Using quantitative complement fixation, they found that the anti-core histone antibodies crossreacted extensively. The anti-H1 preparation did cross react, but only very weakly by comparison. BUSTIN (1978) also reports that JAMRICH et al., (1977) used an anti-calf thymus H1 for immunofluorescence studies on Drosophila salivary gland chromosomes, although the latter authors do not cite either the source or specificity of their antiserum, other than noting it was "reacting with H1".

BUSTIN (1971) has produced evidence, on the basis of chemical modification experiments, that the tyrosine at position 72, which lies in the conserved part of the molecule is important for the recognition of H1 by a number of antisera. However there is also direct evidence for antigenic determinants in a 54 residue long amino terminal fragment of

the molecule (BUSTIN and STOLLAR 1973b; SLUYSER and BUSTIN 1974). Furthermore the ability to produce antisera which discriminate between H1 subfractions (BUSTIN and STOLLAR 1973a) also argues for determinants outside the central region, because sequence studies have shown that this is where most subfraction specific differences lie (JONES et al., 1974).

Although antisera have been raised against Histone 5 which crossreact with histone 1 (MOORMAN et al., 1980, see also EISEN et al., 1981) most anti-H5 antisera have been specifically selected for their ability to discriminate between H1 and H5 (HUANG et al., 1977, SHIMADA et al., 1981).

Possibly, the 405/6 and 401/7/9 preparations are directed only against terminal determinants. Interestingly this preparation was raised against a specific subfraction of histone 1 (subfraction 3). Although it is very probable that the 402 and 147 preparations contain antibodies against the central region of the H1 molecule, this does necessarily mean that they do not also contain antibodies directed against the termini. It would be possible to examine this by making Western blots of H1 molecules which had been proteolytically or chemically dissected (BUSTIN and COLE 1969, BRADBURY et al., 1975, CHAPMAN et al., 1976, ALLAN et al., 1980).

The conserved portion of H1 and H5 is presumably the part which carries out the essential biological function(s) of these molecules. It is interesting to speculate that it might be possible to interfere with this (these) function(s) using antibody preparations such as 402 and 147.

R.11.2 Immunological detection of H1 interaction with DNA supercoils

Histone H1 is known to bind preferentially to supercoiled DNA (VOGEL and SINGER 1975a,1975b). This is probably because H1 binds with high affinity and non-cooperatively to DNA strand crossings (SIMPSON 1978, THOMA et al., 1979, ALLAN et

al.,1980, see also fig.1b in VON MICKWITZ et al., 1979). The evolutionarily conserved central portion of the molecule (R.11.1) has been implicated in this interaction (SINGER and SINGER, 1976, ALLAN et al., 1980, THOMA et al., 1983).

Fig. 55 shows an example of this preferential binding. I^{125} labelled H1 has been added to a mixture of relaxed and supercoiled circular DNA molecules which was then centrifuged in a sucrose gradient. The relaxed and supercoiled molecules have been separated by their sedimentation velocity. The only DNA with which ^{125}I H1 is found associated is the supercoiled species.

A peak of I^{125} activity is also found at the top of the gradient. This material does not appear to be H1. No H1 is immunologically detectable in these fractions, although from the amount of radioactivity present and the known specific activity of the H1 this would be expected. I therefore assume that this represents a meaningless artefact, reflecting the distribution of label and not of intact H1. Some degradation products were present in the radiolabelled material before it was applied to the gradient (SDS-PAGE and autoradiography), but these could only have accounted for at most 40% of the radioactivity found in the artefact peak (probably less because many fragments of H1 retain their immunological activity: BUSTIN and STOLLAR, 1973b, SLUYSER and BUSTIN 1974). Other possible explanations for the appearance of this label are proteolytic degradation during centrifugation, by trace protease present in BSA used as carrier in the gradients, or chemical instability of the iodotyrosine group used for labelling.

By contrast, in agreement with the interpretation that the distribution of the ^{125}I label between the fractions containing the peak amounts of ^{14}C label represents the distribution of intact H1 molecules between relaxed and supercoiled DNA, no H1 could be detected immunologically in the fractions containing relaxed circular DNA molecules, whereas the fractions containing supercoiled DNA gave an

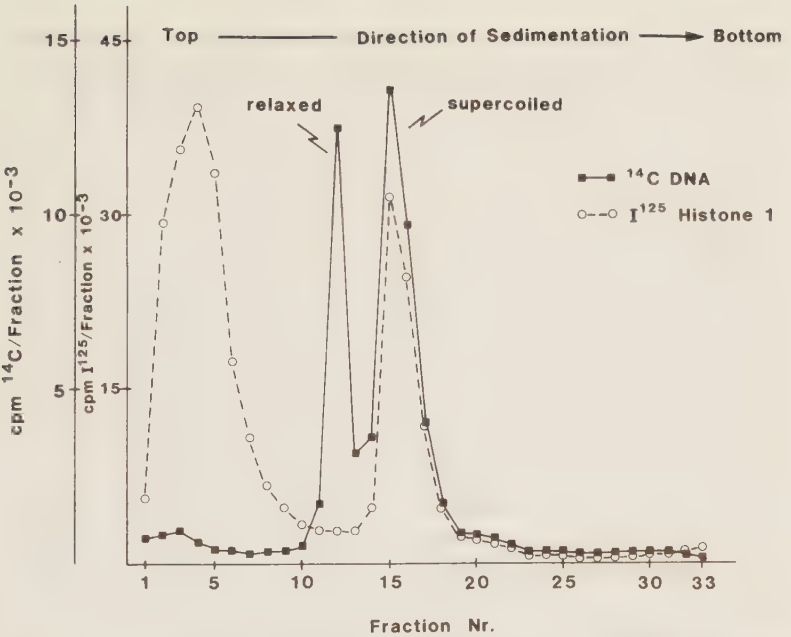

Fig 55 **Preferential association of Histone 1 with Supercoiled DNA** 5 µg ^{14}C labelled ColE1 DNA (5.9 10^4 cpm at the 25% counting efficiency used for this experiment, see M.5.2) was mixed with 3 µg unlabelled H1 and 3.4 10^5 cpm ^{125}I labelled H1 (30 mCi/mg=0.4 mole ^{125}I/mole H1; final specific activity of H1 in gradient = 0.03 mCi/mg H1) in 50 µl buffer and applied to a 5-20% sucrose gradient containg 250 mM NaCl. After centrifugation at 29,000 rpm for 10.75 hr fractions were collected and counted. The symbols designating the distribution of each isotope are indicated on the figure. Other details see M.4.2, M.5.2.

unexpectedly large response in the complement fixation assay. As I show below this large response is due to an enhancement of the assay values for H1 by supercoiled DNA, by a factor of about 20. However, even when allowance was made for enhancement factors, the proportion of H1 binding to relaxed DNA was still negligible compared to the amount found associated with supercoiled DNA, confirming that the distribution of ^{125} H1 between the two forms of DNA is an authentic mirror of the preferential binding of H1 to these different forms.

It was conceivable that the enhancement of the complement fixation assay response using gradient fractions might have occurred because an inhibitor of the H1 anti-H1 reaction was separated from H1 on the gradients. However, the results shown in fig. 56 from experiments using unlabelled and unfractionated H1 and ColE1 DNA strongly suggest that the enhancement effect is the result of an interaction between H1 and supercoiled DNA. The figure shows that, in the presence of supercoiled DNA, the amount of H1 required to produce the same amount of complement fixation, is reduced by a factor 20 compared to that required when H1 is used in isolation. The ColE1 preparation used for these experiments also contained relaxed DNA but, at the H1-DNA ratios employed, almost all H1 would be expected to associate with the supercoiled molecules (cf fig. 55). In the presence of other nucleic acids e.g tRNA (fig. 56), with which H1 also interacts (cf. R.5) or linear DNA (fig 57), the amount of H1 required to give the same assay response was found to be reduced by a factor of only about 2. The assay response using the only other anti-H1 preparation tested (401/7/9) was also enhanced by supercoiled DNA.

Note that, because of the form of the assay response curve, the enhancement factor refers to the **ratio between different amounts of antigen** required to give the same response. Because complement fixation values of zero may be encountered, the two different assay responses obtained using the same amount of antigen, cannot sensibly be expressed as a ratio; the ratio becomes infinite when the unenhanced response falls to zero. The **difference** in assay response using the **same amount of antigen** can however be used as a measure of enhancement (see fig 57).

The minimum amount of H1 which is detectable when enhancement occurs is reduced to less than 4 ng. If enhancement by a similar factor were to occur in the ^{51}Cr release version of the complement fixation assay (R.6.2) then as little as 100 pg of H1 (= 5 femtomoles = 3 10^9

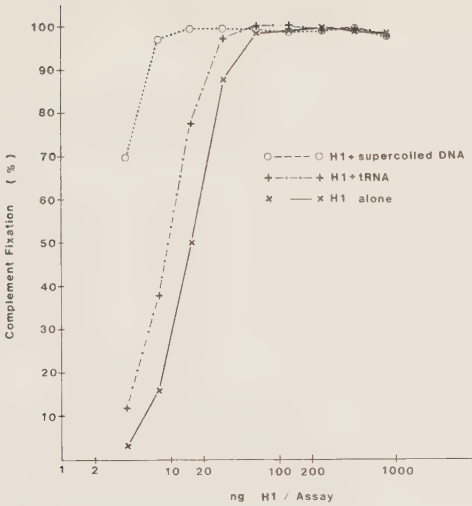

Fig 56 **Enhancement of Complement Fixation assay response to H1 in the presence of supercoiled DNA**

The amounts of H1 indicated on the abscissa and approximately 100 ng/assay of the 402 anti-H1 preparation (cf. R.4.1) were used to titrate complement. The H1 was presented in the form either of H1 alone, H1 mixed with ColE1 DNA (about 50% of which was supercoiled cf. fig. 55) at 0.5 µg H1/µg DNA or H1 mixed with E. coli tRNA at 0.5 µg H1/µg RNA. The solid symbols, whose designations are indicated on the right of the figure are the means of duplicate values. Most duplicates were almost identical, so no ranges are shown. The curves were fitted by inspection. Supercoiled DNA or tRNA alone had no complement fixing activity.

molecules) could be detected, although this was still not as sensitive as solid phase assays (cf. R.7.3.4).

Further evidence that the enhancement effect is attributable directly to a topological feature of the supercoiled DNA is provided by the behaviour of the enhancement effect when supercoiled DNA is titrated with the ethidium ion. Molecules of this dye intercalate into the DNA double helix causing it to wind in a positive direction (cf

JOHNSTON et al., 1979 for a brief description of the meaning of the positive and negative supercoiling effects). When ethidium is used to titrate negatively supercoiled DNA, it first causes it to become unwound and then, as the concentration of ethidium increases further, to become wound in the opposite direction (i.e. positively supercoiled). This effect can be followed experimentally by measuring the sedimentation coefficient of covalently closed circular duplex DNA molecules as a function of ethidium bromide concentration (see e.g. VINOGRAD et al., 1968). Assuming that these molecules are initially negatively supercoiled, as the EtBr/DNA ratio increases from zero, the S values of these molecules falls, reaches a minimum, and then increases, although it never quite reaches its initial value. VOGEL and SINGER (1975b) have shown that the preferential binding of H1 to supercoiled DNA is insensitive to the sense of the winding.

Suppose that complement fixation assay enhancement is attributable only to topological features of the DNA and not, for instance, to the local conformation of particular base pairs. Then, in the course of an ethidium titration the enhancement effect would be expected to first fall, as ethidium causes the DNA molecules to adopt an unwound conformation, and then to rise again as they become positively supercoiled. The uppermost curve in fig. 57a and the two curves in fig. 57b show that this what is observed. At an EtBr/nucleotide molar ratio of 0.05 - 0.06 there is no difference between the level of complement fixation obtained from H1 bound to supercoiled DNA and the amount of complement fixation obtained from H1 bound to linear DNA in the absence of ethidium bromide. This DNA dye-binding ratio is very close to that which has been found experimentally by a number of other investigators to cause complete unwinding of negatively supercoiled molecules with supercoil densities similar to that of Col E1 DNA (CRAWFORD and WARING 1967, VINOGRAD et al., 1968, WANG 1969a,b, 1974, BÖTTGER et al, 1976).

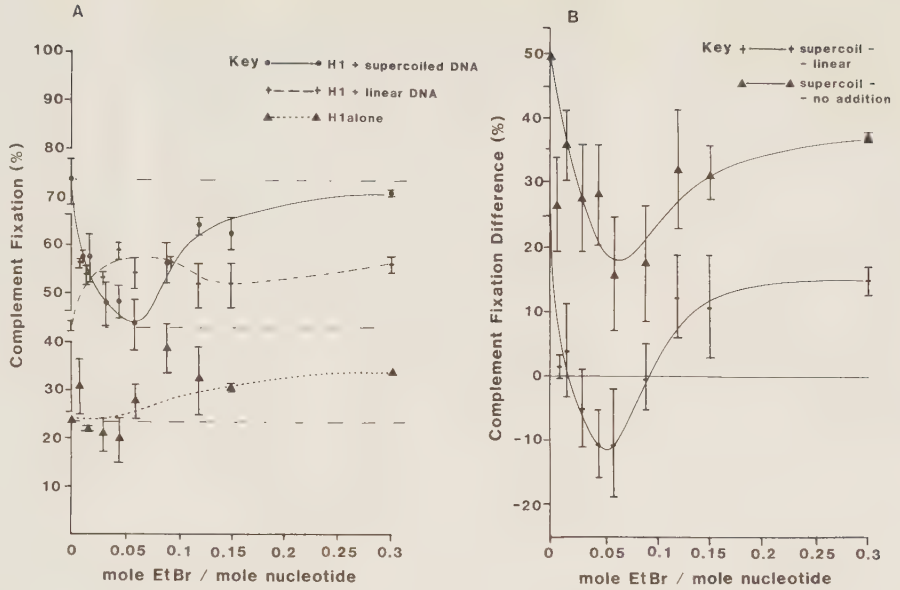

Fig. 57 (see next page for legend)

The interpretation of the experiment shown in fig. 57 is complicated by the effect of ethidium bromide both on the complement fixation assay itself (lowest curve in fig.) and by its interaction with linear (relaxed) DNA. At high concentrations ethidium bromide appears to inhibit the complement reaction, and at low concentrations it appears to enhance the reaction obtained with H1 bound to linear DNA. Although the strands of the linear molecule are free to rotate about one another, and the mean conformation of linear DNA must be relaxed, under some conditions strand crossings can occur at high frequency in linear DNA (VON MICKWITZ et al., 1979). It

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Fig 57 **Ethidium bromide affects the enhancement of complement fixation by H1 and anti-H1 by supercoiled DNA in the the same way as it affects the supercoiling of covalently closed circular duplex DNA molcules.**

Three series of 10 tubes, each tube in the series containing either 0, 0.0045, 0.009, 0.018, 0.027, 0.036, 0.054, 0.072, 0.09 or 0.18 µg ethidium bromide were prepared. To each tube was added 3.7 ng H1 and to each of the 3 series either: no further addition, 50 ng linear DNA (from calf thymus, M.W. > 3 10^6, a kind gift of M.RENZ) or 50 ng circular ColE1 DNA (approximately 50% supercoils). Anti-H1 antibody (402 preparation, R.4.1) was added at the rate of 100 ng/tube and the samples processed for complement fixation in the usual way (M.2.5.1.2.1).

Panel a **Complement Fixation, as a function of ethidium bromide concentration**

The solid symbols whose designations are indicated in the upper right of the panel indicate the means of duplicate treatments. The error bars indicate the range. The dashed horizontal lines indicate the level of complement fixation for each of the three treatments in the absence of ethidium bromide. The curves fitted to each of the three sets of data are interpretative.

Panel b **"Enhancement" - differences in assay response - as a function of ethidium bromide concentration**

The solid symbols show the measured difference between the mean values of complement fixation plotted in panel a: the difference either between fixation in the presence of supercoils and in the presence of linear DNA or the difference between fixation in the presence and absence of supercoiled DNA (designations as shown in the upper right of the panel) is shown. The error bars show the worst case differences between the extremes of the ranges shown in panel a (e.g. greatest supercoil minus least linear and least supercoil minus greatest linear). The fitted curves are interpretative and show the expected effect of ethidium bromide.

is possible that low levels of binding by ethidium might cause the bending of the DNA strand to change (e.g. because of changes in stiffness HOGAN et al., 1983) such that strand crossings occurred more frequently. If strand crossings are the feature of supercoiled DNA to which H1 binds and which are responsible for the enhancement effect then this could provide an explanation for the observed effects. Indeed H1 might stabilise strand crossings, as the inability of nicking-closing enzyme to relax supercoiled DNA in the presence of large amounts of H1 suggests (BINA-STEIN and SINGER 1977). It might be possible to observe such effects by studying the electrophoretic or sedimentation behaviour of linear molecules in the presence of H1 and ethidium.

These complications do not affect the main conclusions to be drawn from this experiment. Enhancement, defined as the **difference** in assay signal between H1-supercoils and either H1 alone or H1-linear DNA, is shown in fig. 57b to follow the predicted course. Enhancement does not return completely to its original level at high ethidium concentrations, but neither does the binding of H1 to positively supercoiled DNA quite reach the level obtained using negative supercoils over the range of ethidium concentrations used in this work (VOGEL and SINGER 1976, fig.3). The sedimentation coefficients of covalently closed negatively supercoiled duplex DNA molecules titrated with ethidium also do not return exactly to their original values (see above and e.g. BÖTTGER et al., 1977 fig. 3).

I have gone into the explanation of the experiment whose results are shown in fig. 57 in some detail, because if my interpretation is correct it means that, in principle, anti-H1 antibodies could be used to probe the conformational state of DNA in chromatin. Such a probe might provide a useful adjunct to antibodies currently being used to study the distribution of Z-DNA in chromatin (NORDHEIM et al., 1981, ARNDT-JOVIN et al., 1983). Exactly how principle could be translated into

practice would depend somewhat on the mechanism by which the enhancement effect occurs. This is unknown, but on the basis of what is known about the properties of H1 and anti-H1 it is possible to outline two more likely possibilities.

I argued in section R.7.3.4.2 that, at least in solid phase assay, a large proportion of the affinity purified anti-H1 preparation appears to be inactive. A possible explanation of this observation might be that antibodies belonging to this part of the total preparation binds only weakly or not at all to isolated H1. However, when H1 binds to DNA strand-crossings, it might undergo a conformational change and expose determinants to which the previously inactive antibody is then able to bind with high affinity. Conformation specific antibodies are known, and since the anti-H1 was prepared from animals immunised with H1-DNA complexes (table 16, R.4.1), it does not seem unreasonable to suppose that the total anti-H1 population might contain members which only bind with high affinity to H1-nucleic acid complexes.

A more trivial explanation might be that proteases present in the complement preparations, with which H1 must be incubated as a necessary part of the assay procedure, might destroy a large proportion of the H1 molecules before they are able to form immune complexes. H1 is known to be extremely sensitive to proteolytic attack (BRADBURY et al., 1975). Complex formation with DNA might protect H1 from such attack, although the protection is more likely to be quantitative than qualitative (ALLAN et al., 1980). However, supercoiled DNA must then be able to protect H1 better than relaxed DNA, again arguing for some qualitative change (?conformational?) in the nature of H1 bound to supercoils. In this case the protease would be the true probe of protein and DNA conformation. It should be possible to decide this question relatively simply by seeing whether the supercoil enhancement effect is observable in other assays in which the influence of protease can be excluded (? solid phase assay?).

This work is not complete. Nonetheless the properties of

this antigen-antibody system that I have explored so far suggest that it could be exploited as a tool to answer some of the many unanswered questions about the mechanism of action of H1 either in chromatin or in model systems (see discussion).

R.11.3 Destruction of H1 antigenic properties by chemical fixation

Table 57 shows that fixation with glutaraldehyde results in the destruction of the antigenic properties of H1 in chromatin.

Glutaraldehyde	% Immunological activity Remaining
0.02% (200 mM)	54
0.04% (400 mM)	35
0.1 % (1 M)	5
0.3 % (3 M)	0.5

Table 57 **Destruction of antigenic properties of H1 in soluble chromatin by glutaraldehyde fixation.**
Chromatin was prepared and fixed as outlined in M.3.3 using the indicated concentrations of glutaraldehyde. Binding of anti-H1 was detected using ^{125}I Protein A as described in M.2.5.2.2. 100% reactivity is binding to unfixed chromatin handled identically. The proportions of bound counts were very similar using three different concentration of antibody, so the data has been pooled. Bound counts have been corrected for unspecific binding (< 0.1% of binding to unfixed chromatin).

Glutaraldehyde concentrations of 0.04% and above are not uncommonly used to bind the H1 in chromatin to DNA for biochemical investigations (OLINS and WRIGHT,1973; CHALKLEY and HUNTER 1975; VARRICCHIO and JAMIESON 1977; LÖFFLER 1978). Still higher concentrations (e.g. 0.2% - 0.5% for 2hr on ice, NEHLS 1977) are used to prepare chromatin for electron microscopic examination (NEHLS, 1977; HOZIER et al., 1977).

The mechanism preventing the immunological detection of H1 in this fixed material is not known. Either H1 might be rendered sterically inaccessible to antibody or the chemical modifications accompanying fixation might destroy the

antigenic determinants on the H1 molecule. Complement fixation by H1 with some antibodies is known to be reduced by maleylation of as few as 2.5 lysine residues/molecule H1 and to be abolished by the modification of 9 (BURNOTTE et al., 1973). Glutaraldehyde also reacts with lysine residues, so chemical destruction is certainly a possibility.

These observations suggest that any attempts to use immuno-electron microscopy to observe representative amounts of histone 1, attached to its *in situ* binding sites, may be doomed to failure if classical methods of fixation are used.

CONCLUDING REMARKS

C.1 Production of monoclonal antibodies in the liquid of ascites tumors and their purification therefrom.

The results presented in chapters R.1 and R.3 of results and discussion document in a fairly straightforward way the steps necessary to grow hybridomas in ascites form and how to obtain purified antibody from the resultant ascites fluid.

The most suprising and novel observation reported is that it is possible to adapt hybridomas of non-Balb/c parentage to growth in Balb/c mice by prior passage in irradiated Balb/c mice. I also show that, provided quality control is carried out, hybridoma ascites can be serially passaged without loss of antibody production.

The adaptation effect appears not to be known to immunologists (NAGY pers. comm.) and the mechanism by which it occurs has not been investigated. Loss of expression of the H2-phenotype of the non-Balb/c "parents" is clearly indicated. The only likely mechanism to account for this which accords with current immunogenetic thinking (NAGY pers. comm.) is chromosome loss.

Although the data of chapter R.1 which allows these conclusions to be made is unequivocal, it was gathered from hybridomas from mice of undefined parentage. It is possible that the effect may not be general. It would therefore be advisable to reproduce the effect using non-Balb/c mice of known pedigree to confirm the authenticity and generality of the effect before reporting on it. Karyotypic analysis could be used to test whether adaptation was occurring by conventional mechanisms.

The finding of adaptation has some useful practical consequences. Apart from not having to irradiate mice (except during adaptation), it means that a laboratory only has to keep large stocks of one mouse strain, the Balb/cByJ strain, with its favorable phenotype (see R.1.1) for the production of ascites, but can use several strains for hybridoma production.

The consequences of this for technology transfer (to underdeveloped countries) should not be overlooked. Production

of hybridomas requires high technology equipment: incubators, sterile hoods, a reliable power supply and so on. However, once hybridomas have been produced and made available in adapted form, their propagation in ascites form requires only the ability to maintain a mouse colony and to have access to a suitable mineral oil (most scientific workers use the pure substance Pristan but many other mineral oil fractions possess similar properties, see references in R.1.1). By using serial passage, and independent of genetic background, laboratories in less well-off countries would be able to produce large amounts of monoclonal antibodies (e.g. for diagnostic purposes) with low investment and production costs.

I would like to draw attention to the observation (R.3.3.3 table 13) that the average yield of antibody in ascites fluid, at least from mice of the strain types used in Tübingen, is only about 2 mg/ml rather than 10 mg/ml (a figure often assumed in the literature, see R.1.1).

In R.3 I have presented schemes for the the purification of monoclonal antibodies of both the IgG and IgM classes to a high level of purity. The IgG purification involves 3 steps: precipitation, gel exclusion chromatography and affinity chromatography. The IgM purification requires only two steps, precipitation and affinity chromatography, but has the disadvantage that the affinity column is not renewable. Both methods have the advantage that they are likely to be insensitive to physical differences between individual monoclonal antibodies (e.g. isoelectric point) and are simple.

The only unsatisfactory aspect of these procedures is that, although affinity chromatography of IgG1 subclass antibodies on protein A is possible (see R.3.4.2), the capacity of the protein A support for this subclass is low. Preparation of the IgG1 subclass requires careful attention to column loading and either multiple, time consuming,

preparations or the use of very large amounts of expensive affinity adsorbent (a column to allow the preparation of the IgG1 from 100 ml of ascites in a single column passage would cost about 5000 DM). This quantitative characteristic of IgG1 binding to protein A does not appear to have been reported previously.

Another method for the purification of IgG which was tried, chromatofocusing, also proved able to separate these antibodies. However, the chromatographic behaviour of IgG on this medium differed greatly from that which was theoretically expected and the deviations were unpredictable. The buffer required to elute antibody from these columns also turned out to have undesirable characteristics (R.3.4.3). Therefore, despite the higher capacity of chromatofocusing columns, this method cannot be recommended for IgG purifications.

Since this work was completed MÜLLER and RAJEWESKY (1983) have reported a method for isolating IgG2 producing variants from IgG1 secreting hybridomas. If this method could be adapted for general use it would offer an alternative strategy for avoiding the problems of IgG1 purification.

In section R.3.5 I draw attention to some differences (e.g. solubility, sensitivity to proteolysis) between individual purified monoclonal antibodies. Failure to take the non-identical properties of monoclonal antibodies into account may lead to difficulties in some experimental situations. (e.g. non-specific binding).

Finally I would like to point out that extensive purification was necessary for many of the studies related to this work. It allowed absolute concentrations of monoclonal antibody to be determined unambiguously. It made possible tests of the reliability of the IgG standardisation assay (see R.8). It allowed ranking of antibody affinities (see R.7.4.4). It made possible the "reverse RIA" technique reported in RISAU

et al., (1983). Finally, it is essential for the reliable production of Fab fragments (R.3.5.5): mouse serum contains a powerful papain inhibitor which is only removed during the affinity chromatography step (unpublished observations of R. HALLMAN).

C.2 Solid phase immunoassay in microtitre plates

In this section I summarise in general terms the major features of the solid phase immunoassay technique using antigen-coated microtitre plate wells. The specific application of this method for the detection and measurement of Drosophila nuclear antigens in sonicated nuclei is discussed in section C.3.

I have described the properties of this method at some length in chapter R.7, because the meaning of the signal generated by antibody binding needs to be properly interpreted if this method is to be used for quantitative measurement. This is especially true for complex mixtures of antigens, for which, as I hope to have shown, a number of problems remain unsolved.

I wish to comment on 8 major characteristics of the method:

1) Assay sensitivity
2) Plate capacity
3) Rate of antigen adsorbtion
4) Non-proportionality between relative assay signal and relative abundance of antigens when mixtures are used to coat the well surface
5) Ranking of antibody affinities
6) Valency of (monoclonal) antibody binding to antigen
7) Indirect technique: amplification of antibody binding
8) Estimation of antibody concentration in hybridoma supernatants

I conclude by making some comments on the application of these findings to other solid phase matrices.

Most of the comments in this section apply equally well to either ELISA or IRMA techniques unless I indicate otherwise.

1)Assay sensitivity

The results of sections R.7.3.4.1, R.7.3.4.3, R.7.4.4.2, R.8.2, R.8.3 and R.9.2.2 either show directly or are consistent with the proposition that indirect immunoassay technique is able to detect a signal from about 10^8 molecules of antigen. This is a practical, measured, limit. The theoretical limit, at least for IRMA, lies still lower (R.7.2). A major factor limiting sensitivity is that either antigen is not always saturated by first antibody (particularly polyclonals) or first antibody is not saturated by detector. The range which can be measured by IRMA, under the conditions used in our laboratories, is less than that covered by ELISA (R.7.3).

In the antigen molecular weight range 10-200 kD, which covers most cellular polypeptides, this corresponds to a mass ranging between about 2 pg and 40 pg. Expressed in moles the sensitivity is:

$$\frac{\text{Nr. molecules}}{\text{Avagadro's Nr.}} = \frac{10^8}{6\ 10^{23}} \simeq 2\ 10^{-16}$$

this brings sensitivity into the range for which the submultiple designation is atto (atto=10^{-18}).

Comparison of the sensitivity I report with that reported by other workers is made difficult by the plethora of reports regarding solid phase assays: IRMA, RIA, ELISA, sandwich assays, all carried out under very different conditions. Volumes, incubation times and/or temperatures and the specific

activity of the labelled detector all vary from investigation to investigation; some factors by an order of magnitude. Even the concept of sensitivity is controversial (EKINS 1979). The result is that immunoassay sensitivities varying over a range of several orders of magnitude have been reported. Furthermore, many investigators express sensitivities only in terms of relative dilution of some substance whose absolute concentration is unknown rather than in absolute terms (see e.g. FOWLER and CHENG, 1983, GRIPENBERG and GRIPENBERG 1983, VAN HEYNINGEN et al., 1983). Nonetheless, of those reports where absolute concentrations can be ascertained, the most sensitive do approach the limits reported here. WEILER (1983) reports several haptenic antigens which he was able to detect at the level of 10^8 molecules using ELISA. KING and KOCHOUMIAN (1979) report the detection of 0.5 ng IgG (10^9 molecules) using ELISA. HUNTER et al., (1982) report the detection of 100 pg alpha-fetoprotein (approx. 10^9 molecules) in IRMA, but with a labelled detector with only half the specificity activity of that used in work reported in this thesis. Similar detection limits have been reported for a homogeneous phase assay, using a panel of monoclonal antibodies with different affinities against the same antigen (WAGENER et al., 1983b).

This sensitivity may be compared to that provided complement fixation, which until recently was one of the most sensitive methods available for measuring antigens in solution. This method could detect about 5 10^{10} molecules in its standard haemoglobin release form (R.6.1) or approximately 10^9 molecules using an improved ^{51}Cr release version (R.6.2). In addition to lower sensitivity, this method required approximately 10 fold more time on the part of the investigator for the measurement of an equivalent number of data points.

In section R.9.3 I suggest some improvements to the specifications of the detector (raising its affinity, specific

activity and the number of sites it recognises) which might allow increases of a factor 10 or more in the intrinsic sensitivity of the assay. This should give a practical detection limit of of 10^7 molecules or 20 attomoles.

The solid phase assay used in the our laboratories, even with the suggested improvements, by no means represents the limits of sensitivity that can be obtained with immunological techniques. Immunofluorescence microscopy, using the naked eye, allows the visualisation of about 10^4 - 10^5 molecules on a single cell (e.g. major cell surface markers such as the thy1, BEECH et al., 1983, or the mouse H2 histoincompatibility antigen, HOLTKAMP et al., 1983). ARNDT-JOVIN et al., (1983), using photon counting techniques have calculated that they can see 500 - 1000 molecules of antigen in an area with the dimensions of a band on a polytene chromosome. H.SAUMWEBER (personal communication) has estimated a similar sensitivity limit using an image intensifying camera (cf. R.10).

The solid phase technique can also be improved by several orders of magnitude by the use of novel detector amplification systems. A sensitivity of 600 molecules has been claimed using a radioactive substrate for the ELISA technique (HARRIS et al., 1979). Fluorescent (ALI and ALI 1983) and luminescent substrates (SIMPSON et al., 1979) also allow substantial improvements.

As I stressed in the introduction to R.7, our method was initially chosen for its convenience and reliability, which allow 100's of samples to be screened in the course of a day, rather than its sensitivity. Nonetheless, for the reasons I go into in more detail in section C.3 in its improved form (10^7 molecules sensitivity) the method would probably be sufficient to allow most regulatory molecules in a nucleus of an organism with a genome the size of Drosophila's to be detected.

2)Plate capacity

The data of section R.7.3.5,1 argues, in agreement with the data of CANTERERO et al., 1980, that the concentration of a pure protein which is required to saturate the plate is one that, in the time allowed for adsorption, would give rise to a monomolecular layer of protein if all molecules bound on contact with the solid phase. However, the proportion of these initially closely packed molecules which are retained in immunologically detectable form thereafter appears to vary with the nature of the plastic from which the microtitre plate is made. However, the retained proteins are esssentially irreversibly bound (see R.7.4.3 and CANTERERO et al., 1980). In absolute terms the maximum amount adsorbed appears to lie in the range of 100 - 300 ng/cm^2 for low M.W. (<200 kD) antigens and in the order of 1 $\mu g/cm^2$ for large particulate antigens in nuclear sonicates.

3)Rate of antigen adsorption

For all practical purposes this can be described by the rate of diffusion of molecules to a totally adsorbing wall. I cannot exclude the possibility that convection may sometimes occur. (see R.7.3.3).

4)Non-proportionality between relative assay signal and relative abundance of antigens when mixtures are used to coat the well surface

The results obtained using defined amounts of an antigen in mixtures (R.7.4.1, R.8.2 and R.8.3) clearly illustrate this principle. The results presented in section R.9.2.2 are also consistent with this notion. The primary effect appears to be mediated by concentration dependent differences in the efficiency with which antigen binds to (or more accurately is retained in immunologically active form on) the solid phase. The effects can be extreme when the aggregate amount of the mixture from which adsorption takes place greatly exceeds the binding capacity of the well wall. The effect cannot be

completely explained by differences in the diffusion rates of the different antigens.

Different detection efficiences by the labelled detector, caused by different numbers of antigenic determinants recognised on the first (usually monoclonal) antibody, may also contribute to non-proportionality (see R.7.4.5, R.7.4.6).

However, under certain conditions, when the amount of protein in a well is near to, or below the well's binding capacity, and sufficient time is allowed for adsorption, the assay probably can be used for the purpose of measuring relative amounts of antigens in mixtures. Even using large amounts of protein and short incubation times, providing the capacity of the plate is not exceeded, relative assay signals may, to a first approximation, reflect antigen abundances. Western blots confirmed the validity of this assumption for the analysis of hnRNP associated antigens (RISAU 1983).

5)Ranking of antibody affinities

In sections R.7.4.4 and R.7.4.5 I measured relative antibody affinities and expressed them, in terms of a molarity, as an equilibrium constant. I should point out that the absolute value of the equilibrium constants thus obtained is only relevant to the system and need not be equal to the equilibrium constant which could be measured in homogeneous phase. The K_{eq} values which I give are empirical values describing the concentration of antibody required to obtain half maximum binding in the solid phase system, using particular volumes of reactants. This is because, strictly speaking, the units in which reactants and products should be expressed in the law of mass action equation, from which K_{eq} is derived, is as activities (the probability of reaction of a molecule) rather than molarities, although in dilute solution molarity and activity are approximately equivalent. I have approximated the activity of the solid phase-bound component in the system as if it had the concentration it would have when in free solution. This is a convenient fiction. The

problem of estimating the true activity of the solid phase bound molecules is that the probability of reaction of any bound molecule with respect to molecules in solution, as a function of distance from the vessel wall, is non-isotropic. Because of random walk effects at the surface the probability of reaction is likely to be dependent upon the geometry of the solid phase itself (see discussion in R.7.2.3.4). Thus, the activity of the solid phase bound component remains unknown, but it would seem unlikely that it would be the same as that observed for equivalent amounts of freely diffusing components. The K_{eq} values of sections R.7.4.4 and R.7.4.5 are therefore only **apparent** K_{eq} values. However, since the measured values fall with the range measured by other workers (see table 19, p.203), they may not be far removed from their solution counterparts.

The apparent K_{eq} values are useful not only because they allow the prediction of the binding of antibody under some specific set of assay conditions, but also because they allow different antibodies to be ranked in order of their affinities. The ranking would be expected to apply to homogeneous phase reactions too, even though the absolute affinities may be systematically displaced with the respect to the numerical values I have calculated.

Although we arrived at our conclusions independently of one another, VAN HEYNINGEN et al., 1983 have adopted an approach formally identical to the one I have described to rank antibody affinities.

6) Valency of (monoclonal) antibody binding to antigen

In section R.7.4.6 I show that there was almost no difference in the binding affinity of the intact P11 antibody and its Fab fragment. If a significant proportion of the intact molecule had bound bivalently, it would have been expected to bind with higher (factor 10 or greater) affinity (see R.7.2.2.2), but this was not detected, arguing that intact antibody binds to this antigen almost exlusively mono-

valently. Although the antigen used for the experiments in R.7.4.6 was probably present in low molecular weight form (?monomer?) the behaviour of P11 was similar when presented in particle form (R.7.4.4.2). It is therefore extremely unlikely that bivalent binding by this antibody is responsible for its satisfactorily high apparent affinity. Since Fab fragments are detectable by detector, albeit with lower efficiency, it follows that a comparison of the affinities of Fab and intact IgG can be used as general method for testing whether intact IgG binds bivalently.

7) Indirect technique: amplification of antibody binding

In sections R.7.4.5 and R.7.4.6 I have presented evidence that the potential molecular amplification of monoclonal antibody varies between 2 and 6 moles of detector per mole of monoclonal antibody, depending on the monoclonal-detector combination. However, it should be borne in mind that amplification will only be realised if detector is saturating the monoclonal antibody being detected. This was clearly not the case in many IRMA determinations (see table 32, p. 337). In ELISA, where higher concentrations of detector could be used without the problems associated with using large amounts of radioactivity, saturation was more probably more nearly approached than in IRMA, but even here detector sometimes turned out to be limiting (R.7.3.4.3) clearly indicating that the potential degree of molecular amplification was not being achieved.

8) Estimation of antibody concentration in hybridoma supernatants

Although not strictly speaking an intrinsic property of the solid phase assay system, the ability to determine, at least roughly, the concentration of monoclonal antibody in supernatant solutions is such an important part of being able to use the method to detect unknown antigens (screening) that

I shall include it here.

In chapter R.8 a very simple method for determining the concentration of supernatant antibody is described. The main point of this method is not that it is very exact, it is not, but that it is sufficiently good to allow hybridomas secreting very high concentrations of antibody to be detected and diluted such that false positives resulting from non-specific binding, which can be a real problem (FRASCH 1981), can be avoided. If some monoclonal antibodies are diluted too much as a result of the inaccuracy of the standardisation assay this will have the effect of increasing the stringency of antibody selection according to affinity. Thus, a few weak antibodies may be lost which might otherwise have been detected. Since most low affinity antibodies are difficult to work with anyway, this loss is not important.

Other solid phases

All the experiments upon which the above conclusions are based were performed on microtitre plates, made out of either PVC or polystyrene. It is difficult to predict to what extent these conclusions will be transferable to other solid phase matrices e.g. nitrocellulose filters. The topology of the microtitre plate well surface is probably simple, to a first approximation it can be considered smooth (see R.7.3.5). The surface topology of a nitrocellulose filter is almost certainly different from that of the microtitre plates, otherwise it would not be possible to explain the very much greater protein binding capacity of these filters (R.HALLMAN and H.SAUMWEBER pers. comm.).

Nitrocellulose filters presumably have a fibrous structure. The area of a web of intermeshed fibres in the volume of a disk of nitrocellulose with unit boundary radius is presumably very much greater than the surface area of a disk of polystyrene of equal radius. While the increased

binding capacity which this increase in surface area causes offers the potential advantage of improving the signal/unit (disk) area ratio, the topological complexity of the filter might also cause problems. The accessibility of the fibre surface at different depths within the filter is not likely to be uniform, and might well vary for particles of different size (i.e. there might be sieving effects). The time course of adsorption would have have to monitored to determine the seriousness of these effects. Although the transport of materials to the boundary of a nitrocelluose sheet could be improved by stirring, the velocity of particles within the filter would almost certainly determined by diffusion. The efficiency with which weakly bound material (e.g. non-specifically bound antibody) is washed from solid phase causes serious problems even in the very open microtitre plate system (see R.7.4.4.1). Leaching from the more complex surface of a nitrocellulose filter might therefore be expected to cause even more serious problems.

C.3 Detection and quantitation of antigens in complex mixtures: limits of sensitivity and its implication for the detection of rare regulatory proteins in eukaryotic nuclei.

The results of chapter R.9, taken together with those from RISAU (1983), indicate that many of the antigens in the Drosophila nucleus against which monoclonal antibodies have been raised by our group (SAUMWEBER et al., 1980) have frequencies of about 10^4 - 10^5 molecules in cultured (?tetraploid, see R.9.1.5) Drosophila cells. In addition the antigens are known to distributed at a large number of sites on polytene chromosomes and are extractable from the nucleus in association with RNA. These are not the characteristics that might be associated with a hypothetical regulatory protein which controls the expression of one or only a few genes within the cell by recognising and binding to specific sequences of DNA. This may be illustrated by comparison with

the properties of known DNA binding regulatory proteins from bacteria, such as the E. coli catabolite repressor binding protein (crp, SAXE and REZVIN 1979, STEITZ et al., 1982), lac repressor (see e.g. KAO-HUANG et al., 1977), or the phage lambda cro and repressor proteins (see PTASHNE et al., 1980, JOHNSON et al., 1981).

Although all the examples referred to above are bacterial, it is possible to make some educated guesses about the frequency with which such rare sequence specific binding proteins might be expected to occur in eukaryotic nuclei. The main problem facing such a protein is the efficiency with which it is able to discriminatorily bind a single (or a few) small sequences of DNA, say about 20 b.p. long, in the presence of a huge excess of non-specific DNA to which it may weakly stick, even if it does not form a stable complex (i.e. weak degenerate binding). The problem is in many senses akin to that faced by an antibody, and there is now evidence (SPERLING et al., 1983) that many antibodies also show degenerate binding, and this would account for much of the "non-specific" background often seen in solid phase assays. The solution of the problem facing a DNA binding protein in nature requires the presence of excess binding protein to ensure that the specific site is found even in the face of non-specific competition. If the weakly bound protein is unable to execute any function at non-specific sites, and is not bound for sufficiently long periods to affect other components of the genetic apparatus, it will not cause "erroneous" regulation , but this does not remove the problem that the amount must be sufficient to allow specifically regulated sites to be found. In the case of the concentrations of specific and non-specific DNA found in an E.coli bacterium this means that approximately 10 copies are required per cell (LIN and RIGGS 1975). For eukaryotes, with larger genome sizes and larger nuclear volumes the required excess is likely to be much larger: the problem has been discussed in some detail by LIN and RIGGS (1975), by WEINTRAUB (1979) and METZENBERG

(1979) who have offered various solutions to the problem, in the sense of devising schemes (e.g. such as masking - making inaccessible - large stretches of "non-specific" DNA) which would increase the efficiency with which a putative regulatory protein might be able to find a binding site.

To date regulatory proteins of this kind remain mainly hypothetical for eukaryotes. There is however, some good genetic evidence for operator - repressor like systems operation in fungi. Four well characterised systems in Neurospora are reviewed in METZENBERG (1979). Details of the Neurospora nit gene system (below) can be found in REINERT and MARZLUF (1975) and WANG and MARZLUF (1979). The genetic evidence for control elements in the purine metabolism system of Aspergillus is described in SCAZZOCHIO and ARST (1978) and in SCAZZOCHIO et al., (1982). A number of systems in yeast (Saccharomyces) have also been characterised (see HUBERT et al., 1983 and references therein).

Despite the impressive genetic characterisations, I know of only two cases, to date, in which attempts to isolate the proteins acting as regulatory elements have been successful. These are of the nit-2 gene product of Neurospora (GROVE and MARZLUF, 1981) and the uaY gene product of Aspergillus (PHILIPPIDES and SCAZZOCHIO, 1981).

Both gene products are positive regulators. The genes whose expression they control will only be expressed when these control proteins are present and bound to a specific cis-acting DNA element associated with the genes they control. In each cases a number (10 or more) of unlinked genes are controlled by the same regulator protein. In these characteristics they are more comparable to the crp control element in E.coli (copy number 1300/cell, SAXE and REZVIN 1979), than the negative lac repressor. There are however differences between the mechanisms of action each protein.

The nit-2 gene product is involved in the control of nitrogen metabolism. In the absence of ammonia in the environment, enzymes are synthesised, which break down and recycle the nitrogen in amino acids. The nit-2 gene product binds to DNA under these conditions. In the presence of ammonia the intracellular glutamine concentration increases, binds to the nit-2 gene product, causes its release from the DNA and the transcription of enzymes for amino acid catabolism is thereby shut off.

The uaY product is required for expression of catabolic enzymes in purine breakdown. However, in contrast, to the nit-2 gene product the uaY product appears to be constituitively bound to DNA. Transcription is switched on by binding of uric acid to the uaY protein and switched off by its absence.

Both gene products were isolated and characterised by virtue of their ability to bind both DNA and effector, glutamine in the case of the nit-2 gene or thiouric acid in the case of the uaY gene. In mutants in which the nit-2 or uaY gene product was expected to be defective, proteins with these specific characteristics were found to be absent, or to have altered binding characteristics. However, it has yet to be shown that the isolated proteins bind preferentially to specific DNA sequences.

The number of copies of each of the two different proteins per nucleus differs greatly. GROVE and MARZLUF (1981) estimate that the nit-2 product is present at frequency of 200 copies/nucleus, whereas PHILLIPIDES and SCAZZOCHIO (1981) estimate a minimum copy number of 10^4 for the uaY product. Since both proteins control the expression of a number (approx. 10) of genes, these figures reduce to 10 and 1000 copies per controlled gene respectively. The reason for this difference is not known, but it is interesting to note that

these frequency classes have their counterparts in E.coli (lac repressor and crp protein, see above). In the context of this work, it is important to note that in solid phase assay a uaY gene product analog in Drosophila would have been detectable (R.9.2.2). In contrast, a nit-2 gene product analog would have lain outside the sensitivity range, at least using crude nuclei as a source of antigen (R.9.2.2). However, it does not seem unreasonable to think that it should be possible to improve the sensitivity of the assay to include this range (R.9.2.3).

Perhaps a more formidable practical problem is providing sufficient antigen to be able to immunise effectively. One of lowest doses of antigen which has successfully led to immunisation required 10^{11} molecules of antigen (LUBEN et al., 1982). Assuming that a similar number would suffice for a putative Drosophila protein, with a frequency of 10^3 copies per nucleus, if unfractionated nuclei were used, the dose would have to be about $10^{11}/10^3 = 10^8$ nuclei/immunisation. This number of nuclei represents 10 mg of nuclear protein, which is a rather high dosage, especially for the *in vitro* system for which this level of sensitivity was described. However, it would be feasible to inject a whole mouse with this amount of protein. Fractionation of nuclei would appear to offer the answer, but here the difficulty lies in ensuring that a hypothetical protein is sufficiently enriched during fractionation. Paradoxically, if nuclei are fractionated in dilute solution, with subsequent shifts of binding equilibria, it might be more efficient to search for regulatory proteins in the soluble rather than in the DNA-bound fraction, in which only a very small percentage of the total in the nucleus would be expected to reside, especially if the off-rate of specific regulator bound non-specifically to DNA were high (LIN and RIGGS 1975, PAINE et al., 1983).

Some alternative approaches might be possible here. The structural features of many of the specific DNA binding

proteins from prokaryotes which have been examined so far have been found to be very similar (OHLENDORF et al., 1983). It might therefore be interesting to construct synthetic peptides corresponding to portions of the binding region of these proteins and to use them as a source of antigen (LERNER 1982, SCHMITZ et al., 1982) in the hope of finding cross-reacting Drosophila proteins of low frequency that contain such sequences. A particular advantage of this method is that the yield of antibody producing cells against many short synthetic peptides is extraordinarily high (NIMAN et al., 1983).

C.4 Quantitative immunofluorescence determinations and possible roles of hnRNP associated proteins in Drosophila polytene chromosomes

The results of chapter R.10 speak for themselves; they show that the relative concentrations of two antigens at different loci on a polytene chromosome can be successfully monitored using the double direct immunofluorescence technique. More importantly, the ratio of concentrations of two antigens at the same locus can be measured in this way. The results indicate that some hnRNP associated antigens (P11, X4, S5) appear together at sites on the chromosome in only a limited number of quantitive ratios to one another (?1:3, 1:2?), whereas the amount of another antigen (T7), although often present at the same sites as the P11 group antigens, does not appear to show any consistent quantitative relationship to them.

The cooccurrence and narrow range of quantitative ratios for the P11/X4/S5 group of antigens suggests that they might be involved in some precise kind of coordinated process. Two imaginable schemes are either that they might occur together in some kind of shortlived particle with a particular stoichiometry or that they might act independently of one another, but possibly sequentially, at a limited number of sites on hnRNA. Either scheme would be compatible with a role

in the cutting or packaging of hnRNA, presuming that most of this takes place while the transcript is attached to its template, for which there is some evidence (BEYER et al., 1981). In contrast, the T7 antigen appears to be involved in some separate process. It seems not always to be associated with RNA, and under some fixation conditions appears to be associated with the nucleoplasm when examined by indirect immunofluorescence (see e.g. fig. 8 of RISAU et al., 1983). Possibly it is involved with transport within the nucleus.

What the quantitative method does not yet allow is an absolute measure of the amount of antigen at chromosomal loci. Even assuming that standardisation of the degree of fluorescent label substitution of the monoclonal antibody (see R.3.4.4) as well as of illumination and of signal amplification were achieved, the level of antigen activity in the system would still have to be determined. The inactivation of antigen by fixation can be a real problem (see R.11) and should not be neglected, at least until some experience with this method of measurement has been gathered. This requires that at least one other independent method of quantitating antigen in the tissue examined by immunofluorescence should be available. Since the copy number of some antigens in Kc cells is now known, it should now be possible to use these cells to calibrate a quantitative immunofluorescence measurement system in absolute terms.

C.5 The use of immunological techniques in determining the biological function of histone H1

I have shown in section R.2 that a variety of immunisation schedules can be used to produce antibodies against calf thymus histone H1. It is clear from the results in section R.11.1 that different rabbits give rise to sets of polyclonal antibodies which are directed against different determinants on the H1 molecule. Some of these determinants

are conserved between evolutionarily widely separated organisms, suggesting that they bind to the conserved central region of the molecule.

The role of H1 in chromatin continues to be something of an enigma. It is perhaps misleading to speak of H1 as a single entity, since it has become clear that there is a large family of related molecules with a similar globular central domain and apparently randomly coiled and highly charged termini wherein the major differences between different members of the family reside (see COLE et al., 1977 for review). It is clear that members of the H1 family play a role in packaging chromatin (FINCH and KLUG 1976, RENZ et al., 1977, THOMA et al., 1979, ALLAN et al., 1981), but no direct evidence has been produced to show whether its presence or absence affects gene expression. There is however circumstantial evidence which suggests that its removal may be required for the expression of genetic activity and that members of the family are involved in the regulation of gene expression.

It appears to be absent in regions both known and presumed to be genetically active (JAMRICH et al., 1977). Numerous tissue specific variants of the family are found in different tissues (COLE et al., 1977) and the synthesis of variants of the $H1_o$ type (PANYIM and CHALKLEY 1969) is known to correlate closely with cell differentiation (EISEN et al., 1981). There is no definitive evidence to show that the appearance of any particular H1 type is necessary for the non-expression of a particular gene. Given the repeated nature of histone genes in many organisms (KEDES et al., 1975, ENGEL and DODGSON 1981), it is possible that the appearance of particular variant might reflect more the state of expression of particular histone loci, rather than any functional role of the particular variants.

However, assuming that H1 is involved in the control of gene expression, it would be reasonable to suppose that the control exerted by these molecules is negative. Certainly

there is a good correlation between the packaging of DNA, the occurrence of H1 and the absence of genetic activity in polytene chromosomes. It is easy to imagine how such control might occur: the packing of DNA by H1 might pose a physical barrier to the passage of RNA polymerase. Various detailed models for hinderance could be imagined. The nucleosomes which H1 arranges might be so tightly packed that passage of RNA polymerase would not be possible. Alternatively chromatin might be sufficiently open, but H1 might act as a topological hinderance by binding DNA crossover points and preventing local DNA unwinding (see GRIGORYEV and KRASHENINNIKOV 1983). These sorts of consideration are very sensitive to the packing organisation of nucleosomes in condensed chromatin that one supposes. A large number of different proposals have been put forward to describe the packing of nucleosomes in condensed chromatin (FINCH and KLUG 1976, RENZ et al., 1977, ALLAN et al., 1980, THOMA and KOLLER 1981). Unfortunately no decisive evidence has been produced to allow discrimination between them, although attempts are being made in this direction (McGHEE et al., 1983).

In the study of H1 itself, much has been made of the differences between various members of the H1 family, but relatively little attention has been paid to the similarity between the central globular portions. Since this region has been conserved evolutionarily, as both sequence (ALLAN et al., 1980) and immunological data (this work and references in R.11.1) attest, it is intuitively appealing to think that the essential biological functions of H1 must rest there. This function would then presumably be the closing of H1 supercoils by binding to DNA crossovers and preventing the relaxation of the two loops round the nucleosome. Apart from the empirical evidence for the binding of the H1 molecule in this position (ALLAN et al., 1980) it would offer a topological mechanism to explain the inhibition of nicking-closing enzyme by H1 (BINA-STEIN and SINGER 1977), rather than one involving

inhibition by direct interaction with the enzyme.

The variable portions of the molecule, the NH_2 and COOH termini, could then be considered as regulatory sequences, although they are also necessary to stabilise higher order structure in chromatin (ALLAN et al., 1982). Their most likely role, in view of the negative control which H1 might be expected to exert, would be to respond to some intracellular signal by causing the removal of H1 molecules from particular portions of chromatin. Different polypeptide termini are presumably required either to recognise particular chromatin regions or to respond differentially to the particular signals in different tissues. Hence, the distribution of H1 subfractions would be expected to vary from tissue to tissue. The relatively simple sequence nature of the polypeptide sequences of some members of the H1 family suggests that the signals causing regulation may be of a relatively non-specific nature: e.g. any long chain polyanion. Whether or not there is any sequence specificity of H1 variant binding, or indeed any localisation within chromosomes remains to be established.

There are several polyanions within the nucleus, for which no role has up to now been found, which might cause the removal of histone H1. HMG1, for example, which has a practically pure polyglutamic acid COOH terminus is known to bind to H1 (SHOOTER et al., 1974, CARBALLO et al., 1983) and is thought to be an essential cofactor for transcription from chromatin (STOUTE and MARZLUFF 1982). Poly-ADP ribose is another enigmatic nuclear entity with suitable characteristics (AUBIN et al., 1983). There are a number of small nuclear RNAs (BUSCH et al., 1982, REDDY and BUSCH 1983) and although the U1 species is thought to be involved in hnRNA processing (LERNER et al., 1980, PADGETT et al., 1983), the role of the others is still not clear (see REDDY and BUSCH 1983 for discussion). Phosphorylation of H1 would offer a convenient way of modulating the removal system (HOHMANN et al., 1976). There

also appears to be a specific, modulated, system for the disposal of unwanted H1 (SUROWY and BERGER 1983).

When H1 has been removed what might keep it absent? It is interesting that the HMG14 and HMG17 proteins, which are known to be associated with transcribed regions (WEISBROD et al., 1980), have been found to bind only to nucleosomes whose DNA is a little longer than the 140 bp core obtained by exhaustive DNAase digestion (SWERDLOW and VARSHAVSKY, STEIN and TOWNSEND 1983). DNA of this length is necessary to allow the DNA strands to cross-over when the ends exits from the coils round the nucleosome. This suggests the intriguing possibility that they might occupy part of the region where H1 might otherwise bind and alter the nature of the H1-DNA interaction. However, it is known that the presence of the HMG14 and HMG17 proteins is not a sufficient condition to cause the disruption of higher order chromatin structure (McGHEE et al., 1982).

The availability of an immunological probe which is able to recognise the binding of H1 to DNA supercoils, and very probably indicates H1 binding to DNA crossovers (section R.11.2), could provide a useful tool for testing some of the above speculations. The initial speculation which should be tested is whether the increased H1 immunological reactivity corresponds to binding to a DNA crossover. If it does, I would predict that increased immunological reactivity should also be seen when H1 is presented in the form of chromatosomes (nucleosomes with a full complement of histones and containing two full turns of DNA round a protein core, SIMPSON 1978). If this condition is fulfilled it should be possible to test, by its influence on the enhancement effect, whether any of the agents which I have suggested above is capable of interfering with binding of H1 to a crossover. It would be possible to do this even if the H1 were displaced from the crossover, but remained bound to the DNA (see e.g. AUBIN et al., 1983).

Apart from this possible application, the finding of

section R.11.2 might be considered by some to be intrinsically interesting. Although conformational features of DNA have been detected by immunological techniques (MÖLLER et al., 1982), to my knowledge my findings are the first report of a topological feature of DNA being detected by immunological techniques.

SUMMARY

The results reported in this thesis are organised into two parts (I and II). Part I, which forms the major part of the work, is exclusively concerned with methodological aspects of antibody production, purification and measurement. Part II gives examples of how the antibodies produced and assayed using the methods described in part I can be used to answer topical questions about the composition and function of the eukaryotic nucleus. The development or testing of the methods described in part I was essential to obtain the results reported in part II. Many of the methods described in part I will be of use to other investigators wanting to use immunological techniques to analyse cell structure and function.

Reasons for the choice of monoclonal and polyclonal antibodies used in this study are discussed in the foreword to the "Results and Discussion" section of the thesis (p. 96).

Each part of the "results and discussion" section is divided into 3 major subsections (A,B and C).

Part IA describes the production of antisera.

The adaptation of histoincompatible hybridomas to growth as antibody producing ascites tumors in Pristane-primed, but otherwise untreated, Balb/c strain derived mice by prior passage in irradiated mice is described (R.1 p.98). The origin of the histoincompatibility of the hybridomas used, and the reasons for wishing to use Balb/c strain derived mice are discussed. It is shown that the ascites can be serially passaged. Although occasional instabilities are observed these can be countered by regular quality control. The yield of antibody from ascites sera is between 1-4 mg/ml.

It is shown (R.2 p.114) that, in rabbits, the "poor" antigen histone H1 gives rise to positive antisera in almost all animals, given sufficient immunisation, although titres vary greatly. There is some indication (R.4.2 p.177) that the

immune response of rabbits to this antigen may be stereotyped.

Part IB describes antibody purification and characterisation.

The necessity of working with purified antibodies for some purposes is argued in section R.3.1 (p.120). Theoretical and practical difficulties associated with the purification and use of monoclonal antibodies are discussed in section R.3.2 (p.122).

A very simple method for the purification of mouse monoclonal antibodies is described (R.3.3 p.125). Only ammonium sulphate precipitation and gel filtration on Sephacryl S-300 is required. The rationale behind this purification scheme, the neccessity of an ammonium sulfate precipitation step and some problems associated with it are discussed. Although the antibodies produced by this simple method are probably sufficiently pure for many practical purposes, they still contain a number of minor contaminants including a papain inhibitor, precluding the production of Fab fragments. For such purposes further purification is neccessary. Methods for purifying IgG and IgM to near homogeneity are therefore presented (R.3.4 p.136). Affinity purification of IgM on conA and of IgG on protein A is shown to be very effective, but it is shown that care must be taken with IgG1 subclass antibodies to avoid exceeding the capacity of the affinity support. It is shown that it is also possible to purify mouse antibodies by the method of chromatofocusing (PHARMACIA), but that the method has many disadvantages.

Purified antibodies are characterised by isoelectric focusing and shown to be homogeneous, apart from their intrinsic microheterogeneity (R.3.5.1 p.157). Some hybridomas are shown to produce more than one antibody: the causes and consequences of this are discussed. Purified monoclonal antibodies are shown to differ in their solubility properties,

the ease with which they may be chemically modified and their susceptibility to proteolysis (R.3.5.3 - R.3.5.5, p.168-193). These examples of differences point to the inadvisability of treating the mouse IgG class of molecules as if its members had uniform properties. Serious consideration must be given to physico-chemical differences, at least at the subclass (isotype) level, if not at the level of individual antibodies (R.3.2.1, p.122) if monoclonal antibodies are to be usefully used.

Preparation of pure monospecific, polyclonal rabbit anti-H1 by affinity chromatography is described (R.4.1 p.174). Some dangers of the use of DEAE-cellulose and DEAE affi-gel blue for antibody purification are documented. The isoelectric focusing spectrum of the preparations is described (R.4.2. p.177).

Part IC deals with several aspects of immunological assay methods.

Some peculiar properties of the histone 1-anti H1 system, which prevent its assay by immunoprecipitation, are documented (R.5 p.182).

Assay of H1 by complement fixation, the sensitivity of this method and a chrome release version thereof are described (R.6 p.188). The reasons for including a description of this now obselete method are discussed in the foreword to this section.

The properties of a solid phase assay using the walls of microtitre plate wells as the solid phase support for antigen are described (R.7 p.193 and R.8. p.351). Assay nomenclature (RIA, IRMA, ELISA) is discussed (R.7.1 p.196). Theoretical aspects of binding reactions, and of the differences between

homogeneous and heterogeneous phase reactions are considered (R.7.2 p.197). The range of binding constants ascribed to monoclonal antibodies which have been characterised to date (R.7.2.1.2 p.201) is presented. The drastic increase in antibody affinity which can be caused by bivalent antibody binding is discussed and shown to be unlikely to be important for the detection of rare antigens (R.7.2.2 p.214). The processes governing the rate of adsorbtion of molecules in solution to the well walls are considered (R.7.2.3 p.225). Complications resulting from heterogeneity of antibody (R.7.2.4 p.239) or antigen (R.7.2.5 p.241) are considered. Using the results of a model H1-anti H1 system (R.7.3 p.242) and those of the mouse IgG-anti IgG system (R.8 p.351), the intrinsic and practical limits to the sensitivity of the assay using both radiolabel and enzyme-linked detection methods are estimated and discussed. Attention is drawn to the tendency of phosphatase derived anti-mouse antibody to give rise to artefactual signals (R.7.3.1.2.5 p.254). Under the conditions of assay used in our laboratory, the lower limit of detection for antigen is about 10^8 molecules (R.7.3.4 p.271, R.8.3 p.359). The capacity of the well surface for retaining antigen is shown to vary between plates made of different plastics. The capacity of PVC plates is shown to be approached by the binding of a monomolecular layer of antigen, approx. 240 ng/well or 1.4 10^{10} molecules/mm^2 for a molecule with the dimensions of IgG; it is lower for polystyrene plates and affected by temperature (R.7.3.5.2 p.288). The adsorption rate of antigens to the well surface is shown to be approximated by the the rate of diffusion of the antigens (R.7.3.3 p.267).

A by-product of the model studies was the observation that all affinity-purified polyclonal antibodies used bound inefficiently to their antigens (R.7.3.4.1 p.271, R.7.4.5 p.331, R.8.3 p.359), suggesting that, despite affinity purification, they may have contained a substantial proportion of low affinity antibody.

When an antigen in a mixture of proteins is used to coat

a well surface, it is shown that, under certain conditions, antigen may be selectively excluded from adsorbtion. Selective exclusion is shown to be dependent upon the concentration of the mixture used as a source of antigen (R.7.4.1, R.7.4.2, R.8.2). The capacity of microtitre plate for protein in crude sonicated preparations of Kc cell nuclei, which contain much particulate material is about 1 µg (R.7.4.3 p.310).

A major factor interfering with the specific assay of antigen by monoclonal antibodies is shown to be non-specific binding when high concentrations (> 10 µg/ml) of monoclonal antibody are used. The use of low concentrations of antibody (< 1 µg/ml) is essential to avoid artefacts from this source. It is shown that even when these low concentrations of antibody are used against hnRNP associated antigen in crude nuclear sonicates that the affinity of most of the antibodies studied was sufficient to saturate all antigen binding sites (R.7.4.4 p.317). For one antibody it is shown that the affinity of the monoclonal antibody is an intrinsic property of the combining site rather than a consequence of bivalent binding (R.7.4.6 p.342). The amount of immune complex derived using nuclear sonicates as a source of hnRNP-associated antigen is sufficiently low to enable accurate estimates of the signal generated by saturating amounts of labelled anti-antibody to be made (R.7.4.5 p.331). By making reasonable assumptions about anti-antibody:monoclonal stoichiometry (R.7.4.5 p.331, R.7.4.6 p.342, R.8.3 p.359), the known specific activity of the anti-antibody allows quantitative estimates of adsorbed antigen.

The major uncertainty in estimating the amount of a particular antigen in a nucleus is then the efficiency with which can be adsorbed from a crude protein mixture. Unfortunately this appears to vary from antigen to antigen (e.g. because of selective exclusion, see above) and to be sensitive to factors such as being part of a particle, which may in turn be sensitive to the ionic conditions under which adsorption takes place (R.7.4.2 p.304). This remains the major

stumbling block to using solid phase assay for accurate determination of individual antigens in mixtures and is addressed again in chapter R.9 (p.368).

The binding of one (out of four tested) antibodies against anti-hnRNP associated proteins was temperature and salt sensitive (R.7.4.7 p.346), indicating another potential source of error which must be controlled for.

The IgG content of hybridoma supernatants varies by a factor of more than 100 and extends into the range where non-specific binding effects are a nuisance (see above). This can create problems when screening hybridoma supernatants. A very simple solid phase assay, for the determination of monoclonal antibody in supernatants is presented (R.8.2. p.353). The assay uses less than 1 µl supernatantant and is accurate to within a factor 2 - 5 (R.8.3 p.359). The accuracy of the method is sufficient to allow antibody concentrations in supernatants to be adjusted to avoid non-specific effects. The assay makes use of a counterintuitive dilution step to reduce selective exclusion of the monoclonal antibody during the adsorbtion step thus bringing the assay response into the useful range (R.8.2 p.353). The limited accuracy of the method is at least in part caused by anti-antibody recognising different determinants on different monoclonals, although other factors may also have an effect (R.8.3 p.359).

Part IIA describes quantitative estimates of hnRNP associated antigens in the Drosophila Kc cell nucleus.

The macromolecular composition (DNA, RNA, protein) of exponentially growing cells, nuclei and hnRNP extracts were measured (R.9.1 p.368). I have been unable to find any comparable published measurements for insect cells. Estimates of precision and reliability of the measurements are given. The Kc cells used by our group until 1983 are probably

tetraploid (R.9.1.5 p.376). It is noted that the RNA and hnRNA content of the Kc cell nuclei is probably greater than that of vertebrate cells by a factor 2 - 5 (R.9.2.1.1 p.402). The protein content may also be greater.

From a knowledge of the macromolecular composition of the nucleus, it is possible, on the basis of simple assumptions about protein:RNA stoichiometries and the relative proportion of packaging to non-packaging proteins in isolated hnRNP, to make rough estimates of the frequency of the common hnRNP-associated non-packaging proteins, approx. 10^5/nucleus. The immunological estimates (solid phase assay data) will agree with the non-immunological estimates if antigen adsorbtion efficiencies in the order of 1 - 5% from crude nuclear sonicates and of 15% from hnRNP fractions purified by centrifugation (R.9.2 p.403) are assumed. On the basis of these assumptions the solid phase assay data is also in good agreement with estimates for P11 and X4 antigens made by RISAU (1983, Ph.D thesis). For the S5 antigen the agreement with the RISAU data is less good. Possible reasons for this discrepancy are discussed.

It is shown that, using current solid phase assay techniques, antigens with a frequency of 10^3 copies/nucleus should be detectable. Improvements are suggested, which might realistically be expected to allow the detection of antigens with a frequency of between 1 - 10 copies per nucleus (R.9.3 p.410).

Part IIB describes a quantitative comparison of the distribution of hnRNP associated antigens on Drosophila polytene chromosomes.

The constancy of the relative concentration of pairs of antigens at different loci on polytene chromosomes is made by comparing the relative fluorescence intensity of the red and green fluorescence from chromosomes simultaneously stained

with pairs of directly differentially labelled monoclonal antibodies. The image processing method used for comparison is described (MIP method, R.10.3 p.424), as is the optimal antibody concentration for chromosome staining, which is different from that required for solid phase immunoassay (R.10.2 p.420). Direct labelling of monoclonal antibody is shown not to measureably affect its antigen binding properties (R.10.2) and control experiments show that the MIP method fulfils the criteria set for it (R.10.4 p.428).

Under normal conditions (i.e. not heat shock), the occurrence of the P11, X4 and S5 antigens (P11 group) is qualitatively identical, and quantitative variation in the ratio with which the antigens occur relative to one another appears to be discretely distributed (e.g.2:1, 1:1, 1:2 etc.), suggesting some kind of functional association between these antigens. By contrast, although the T7 antigen does occur at loci where P11 group antigens are found, it also occurs independently of them. In addition the variation in ratio between the T7 antigen and those of the P11 group antigens appears to vary nearly continuously rather than discretely. Taken together these two observations suggest that T7 antigen may involved in a different function from that of the P11 group antigens (R.10.5 p.432). Variations in the distribution of antigen on chromosomes obtained from different larvae are interpreted as reflecting the dynamic nature of the complexes with which these antigens are associated (R.10.6 p.440).

Part IIC describes some immunological properties of histone H1 which may contribute to an understanding the function of this histone.

The cross reaction of different anti-H1 preparations with the H1's of different species is reported. The patterns of reaction indicate that different anti-H1 preparations must recognise different antigenic determinants (R.11.1 p.446).

The immunological reactivity of H1 complexed with supercoiled DNA, to which H1 binds preferentially, is shown to be increased by an order of magnitude in the complement fixation assay. Evidence is presented that a topological feature of the DNA (?DNA cross-overs) must be responsible for this effect. Possible mechanisms for the effect are discussed (R.11.2 p.451).

It is shown that glutaraldehyde fixation can lead to the almost complete loss of the immunological reactivity of H1 in chromatin (R.11.3 p.461).

In the "Concluding Remarks" chapter (C.1 - C.5, p.464 - p.487), some of the wider implications of the above findings are discussed.

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Short life history

Personal details

Birth	7. 7. 1949 London, England
Father	John Symmons
Mother	Elizabeth Mary Brown Symmons (née Craig)
Nationality	British
Personal Status	Married to Noémi Lukács (née Lukács) 1 daughter

Education and Employment

1957 - 1963	Colet Court preparatory school Hammersmith, London, England.
1963 - 1967	St. Paul's School, Hammersmith, London, England.
1967 - 1970	Honours degree course in agricultural botany at Reading University, England.
1970	Batchelor of Science (B.Sc.) degree of Reading University.
1970 - 1974	Part-time study of biochemistry at Chelsea College, University of London, England.
1970 - 1972	Employment as research assistant in the biophysics department of Chelsea College.
1973 - 1977	Employment as technical assistant in the department of physical biology (head Dr. F. Bonhoeffer) at the Max Planck Institute (MPI) for Virus Research, Tübingen, Federal Republic of Germany (FRG).
1974	Master of Science (M.Sc.) degree of London University.

1975 - 1981	Immatriculated at the University of Tübingen, FRG.
1975 - 1983	Research for doctoral thesis, under the supervision of Dr. F. Bonhoeffer, in the department of physical biology, MPI for Virus Research, Tübingen.
1977 - 1979	Stipendium from the Max Planck Society.
1979 - present	Employment, first as technical assistant, then scientist in the department of physical biology, MPI for Virus Research, Tübingen.

My academic instructors were:

(Reading)	(London)	(Tübingen)
Prof. A.H.Bunting	Prof. H.Baum	Prof. A.Gierer
Prof. V.Heywood	Prof. R.B.Beechey	F.Bonhoeffer
Prof. T.Harris	Prof. R.V.Brooks	A.Klein
Prof. E.W.Russell	P.J.Butterworth	M.Renz
Prof. C.Tyler	D.F.Evered	
Prof. A.H.Watson	G.S.Hall	
P.Cartwright	K.R.Harrap	
M.I.Crichton	C.T.Leese	
L.K.Crowe	C.W.Parr	
R.N.Curnow	M.C.Perry	
D.S.Drennan	T.I.Steenson	
M.W.Dick	W.P.Williams	
J.F.Elston	J.M.Wrigglesworth	
K.A.Hassall		
F.B.Hora		
J.K.Jones		
D.M.Keith-Lucas		
J.P.Moss		
H.Owen		
B.Pickersgill		
C.J.Smith		
R.W.Snaydon		
J.Snow		
R.S.Tayler		
R.M.Wadsworth		